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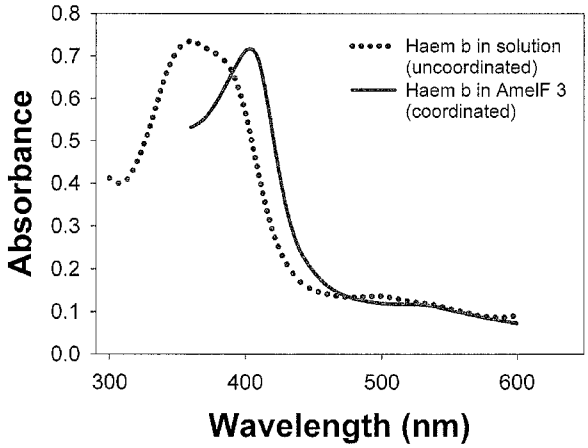
(54) Title: METALLOPROTEIN COMPOSITIONS

Figure 2



(57) Abstract: The present invention relates to compositions comprising: a polypeptide, wherein at least a portion of the polypeptide has a coiled coil structure; and a chelate comprising a chelating agent and a metal ion; and wherein the chelate is bound to at least one amino acid of the polypeptide. In a preferred embodiment the polypeptide is a silk fibroin, wherein at least a portion of said silk fibroin has a coiled coil structure.

METALLOPROTEIN COMPOSITIONS

TECHNICAL FIELD

[001] The present invention relates to compositions comprising a polypeptide and a chelate comprising a chelating agent and a metal ion, and uses thereof.

BACKGROUND OF INVENTION

[002] Detecting the presence of, and levels of, molecules in mixtures of interest is of great commercial importance.

[003] As a consequence, there have been efforts to develop methods for detecting and monitoring levels of molecules of interest using molecules able to interact with the molecules of interest. Some of the most promising of these methods involve the use of a biosensor. Biosensors are devices capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element. The biological recognition element of a biosensor determines the selectivity, so that the molecule which has to be measured generates a signal. The selection may be based on biochemical recognition of the molecule where the chemical structure of the molecule is unchanged, or biocatalysis in which the element catalyzes a biochemical reaction of the molecule. A transducer translates the recognition of the biological recognition element into a semi-quantitative or quantitative signal. Possible transducer technologies are optical, electrochemical, acoustical/mechanical or colorimetrical. The optical properties that have been exploited include absorbance, fluorescence/phosphorescence, bio/chemiluminescence, reflectance, light scattering and refractive index. Conventional reporter groups such as fluorescent compounds may be used, or alternatively, there is the opportunity for direct optical detection, without the need for a label.

[004] Because the biological recognition element of a biosensor determines the specificity, proteins are of great interest for use in biosensors, since there are a wide variety of protein domains known to bind molecules of interest with high specificity and sensitivity, for example monoclonal antibodies and derivative single-chain variable fragments (scFvs), enzymes, viral proteins, protein aptamers etc.

[005] However, the characteristics of many proteins make them less amenable to use in biosensors. For example, many proteins become unstable after purification, undergoing irreversible conformational changes, denaturing, and loss of biochemical activity.

[006] The immobilisation of proteins within or onto materials also presents problems. For example, proteins are frequently immobilised onto surfaces by non-specific covalent bonding and therefore can exist in a large number of possible orientations, for example, with some proteins oriented such that their binding or active sites are exposed whereas others may be oriented such that there active sites are not exposed, and thus not able to undergo selective binding reactions with the molecule of interest. In addition to orientation, protein density may also be poorly controlled. Proteins are also subject to time-dependent denaturing, denaturing during immobilization, and leaching of the entrapped protein subsequent to immobilization. Furthermore, immobilisation may limit contact between the protein and the diagnostic molecule of interest.

[007] Therefore, there is a need in the art for stable protein biosensors that can be formed into materials, or incorporated into or onto materials.

SUMMARY OF INVENTION

[008] The present inventors have surprisingly found that polypeptides comprising a portion with a coiled coil structure, such as coiled coil silk polypeptides, a chelating agent, such as a macrocycle, and metal ion can be used to provide compositions and materials having desired characteristics, for example for use as biosensors and the like.

[009] Thus, in one aspect, the present invention provides a composition comprising; a polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; and a chelate comprising a chelating agent and a metal ion; and wherein the chelate is bound to at least one amino acid of the polypeptide. In some embodiments, the chelating agent is bound to at least one amino acid of the polypeptide. In some embodiments, the at least one amino acid residue bound to the chelating agent is a charged amino acid residue. In some embodiments, the metal ion is bonded to at least one amino acid of the polypeptide by a co-ordinate bond. In

some embodiments, the at least one amino acid bound to the metal ion by a co-ordinate bond is a Tyr, Cys, His, Met, Lys, Glu or a non-natural amino acid.

[0010] In some embodiments, the portion of the polypeptide that has a coiled coil structure comprises at least 35 amino acids, or at least 63 amino acids.

[0011] In some embodiments, at least 20% of the amino acids in the coiled coil structure are alanine residues.

[0012] In some embodiments, the chelating agent comprises a ring of atoms. In some embodiments, the chelating agent is selected from the group consisting of porphryins, corrins, chlorins, corphins, porphines and phthalocyanines.

[0013] In some embodiments, the metal ion is an ion of a transition metal, alkali earth metal or p-block metal. For example, the metal ion may be selected from the group consisting of an ion of Fe, Sn, Cd, Cr, Mn, Co, Cu, Ru, Zn, Mg, Sc, Ru, Rh, Os, Ag, Pd, Zn, Re, Pt, Ti, V, Ni, Mo, Tc, W and Ir. In an embodiment, the alkali earth metal is Mg.

[0014] In some embodiments, the composition of the present invention is capable of binding a target compound. In some embodiments, the composition of the present invention comprises a binding site for a target compound. The target compound may be selected from the group consisting of oxygen, carbon monoxide, carbon dioxide, hydrogen peroxide, compounds having an atom of P, S, or N, and mixtures thereof. In some embodiments, the target compound is NO.

[0015] In some embodiments, the composition comprises more than one polypeptide.

[0016] In another aspect, the present invention provides a material comprising a composition of the present invention, wherein the polypeptides are crosslinked by ionic bonds, Hydrogen-bonds, covalent bonds or a combination thereof and the material is insoluble in water. The material may be in the form of a silk fibre, film, powder or sponge.

[0017] In yet another aspect, the present invention provides a copolymer comprising a composition of the present invention and a further polypeptide, wherein

at least a portion of the further polypeptide has a coiled coil structure. In some embodiments, at least some of the polypeptides are crosslinked. In some embodiments, at least some of the residues of the polypeptides are covalently crosslinked.

[0018] In yet another aspect, the present invention provides a sensor for detecting a target compound comprising a composition, material or copolymer of the present invention. In some embodiments, the composition, material or copolymer comprises a binding site for the target compound, and wherein binding of the target compound results in a detectable change. The detectable change may be a change in colour, spectrophotometric, fluorescent or electrochemical change. In some embodiments, the spectrophotometric change is a change in the Soret peak. In other embodiments, the spectrophotometric change is a change in at least one spectrophotometric peak with a wavelength between 500 and 600 nm.

[0019] In yet another aspect, the present invention provides a method of binding a target compound, said method comprising the steps of (a) providing a composition, material or copolymer of the present invention and (b) contacting said composition, material or copolymer with a target compound under conditions for binding said compound to said composition. In some embodiments, the method further comprises detecting binding of the target compound by detecting a change in the composition and/or target compound upon binding.

[0020] In yet another aspect, the present invention also provides a method for producing a biosensor, said method comprising providing a polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; and contacting the polypeptide to a chelate comprising a chelating agent and a metal ion under conditions for binding said chelate to at least one amino acid of said polypeptide. In some embodiments, the chelating agent is bound to at least one amino acid of the polypeptide. In some embodiments, the at least one amino acid residue bound to the chelating agent is a charged amino acid residue. In some embodiments, the metal ion is bonded to at least one amino acid of the polypeptide by a co-ordinate bond. In some embodiments, the at least one amino acid bound to the metal ion by a co-ordinate bond is a Tyr, Cys, His, Met, Lys, Glu or a non-natural amino acid.

[0021] In a further aspect, the present invention provides a method of detecting nitric oxide, the method comprising (a) contacting a sample with a composition or material of the invention, (b) determing if step (a) results in a detectable change in the composition or material, wherein a detectable change indicates that nitric oxide is in the sample. In an embodiment, the chelate is haem *b*.

[0022] Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise.

[0023] The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

[0024] Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

[0025] As used herein, the term about, unless stated to the contrary, refers to +/- 10%, more preferably +/- 5%, of the designated value.

[0026] Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

[0027] The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF DRAWINGS

[0028] **Figure 1** shows evidence for the binding and coordination of haem *b* within honeybee silk sponges. A solution of haem (green colouration) is added drop-wise onto a honeybee silk sponge (A; top panel) or silkworm silk sponge (B; bottom panel).

The honeybee silk sponge changes to a red colouration, while no colour change occurs in the silkworm silk sponge. The haem-induced colouration of the honeybee sponge remains after washing with 70% methanol, whereas the green haem *b* colour is washed out of the silkworm silk sponge. This figure demonstrates binding and coordination of a chelate to a polypeptide of the present invention.

[0029] **Figure 2** shows evidence for the binding and coordination of haem *b* within honeybee silk films. UV/Vis spectra obtained from an AmelF3-haem *b* composite film generated from honeybee silk protein AmelF3 show a sharp Soret peak at 410 nm (solid line). In contrast the spectrum from uncoordinated haem *b* in aqueous solution shows a broad Soret peak below 400 nm (dotted line). The shift in the Soret peak indicates that the haem centre is coordinated to a residue in an AmelF3 polypeptide of the silk protein film. This figure demonstrates binding and coordination of a chelate to a polypeptide of the present invention.

[0030] **Figure 3** shows a comparison between the UV/Vis spectra obtained when the molar ratio of haem *b* to AmelF3 was varied. As the concentration of haem *b* relative to the protein was increased, the Soret peak broadened and shifted to lower wavelengths indicating an increase in the amount of uncoordinated haem. At ratios of 1:1 haem:AmelF3 or lower, the Soret peak at 404 nm indicates that all the haem is coordinated. As haem:AmelF3 ratios increase, the Soret peak widens and shifts to 398 (insert), indicating an increase in the amount of uncoordinated haem. The stoichiometry demonstrated in this figure indicates specific saturable binding of a chelate to a polypeptide of the present invention.

[0031] **Figure 4** shows that polypeptides of the present invention coordinate the metal ion of the chelate through a tyrosine residue. Raman spectra of AmelF3-haem *b* (middle trace) shows a broad feature centred at 594 cm^{-1}. Raman spectra of AmelF3 (top trace) and haem *b* trace (middle trace) do not display a broad feature centred at 594 cm^{-1}. These spectra are consistent with a tyrosine residue coordinating to the haem centre.

[0032] **Figure 5** shows binding and coordination of a chelate to a polypeptide of the present invention both in solution and in a material. The figure also shows that Tyr76 of AmelF3 is important for co-ordinating haem *b*. A. Comparison between the

UV/Vis spectra obtained when haem *b* was added to silk protein films generated from recombinant honeybee silk protein AmelF3 and AmelF3 with a mutation of tyrosine 76 to alanine (AmelF3.Tyr76Ala). The broad Soret peak at 395 nm indicates that the modified honeybee silk does not coordinate to haem *b*. B. Aqueous solutions of AmelF3 with a mutation of tyrosine 76 to histidine (AmelF3.Tyr76His) and haem *b* in a 1:1 ratio. The sharp peak at around 400 nm, known as the Soret peak, is indicative of the coordination of an amino acid residue to the iron haem centre. The data show that the metal ion is coordinated in solution and in film.

[0033] **Figure 6** shows UV/Vis spectra of silk films generated from recombinant green ant silk proteins (GA1-4) after haem *b* was leached in. This data shows that for GA1 and GA3, a strong signal was observed at around 400 nm demonstrating that these proteins strongly bound haem. This data shows that other polypeptides, besides honey bee silk polypeptides, having a coiled coil structure are capable of binding a chelate comprising a chelating agent and a metal ion.

[0034] **Figure 7** shows UV/Vis spectra of silk films generated from recombinant praying mantis silk proteins after haem *b* was leached in. A strong signal was observed at ~400 nm demonstrating that these proteins strongly bound haem. This data show that other polypeptides having a coiled coil structure are capable of binding a chelate comprising a chelating agent and a metal ion.

[0035] **Figure 8** shows the chelator of the chelate may be varied. The non-porphyrin chelator cobyrnic acid (dicyanocobyrinic acid heptamethyl ester) was added to silk protein films generated from recombinant honeybee silk protein AmelF3. The shift in the maximum absorbance of the sharp peak from 370nm to 360 nm, upon addition of the chelator to the silk film, is indicative of the coordination of an amino acid residue to the cobalt centre when dicyanocobyrinc acid heptamethyl ester is leached into the AmelF3 film.

[0036] **Figure 9** shows UV/Vis spectra obtained when silk protein films generated from recombinant honeybee silk protein AmelF3 were reduced and then re-oxidised, showing that the reaction can be reversed. The oxidation state of the haem centre is determined from the UV/Vis spectral shifts. With reduction the Soret peak shifts from

410 nm to 420 nm and an increase in peak at 550 nm observed. This data shows compositions of the present invention are capable of being reversibly reduced.

[0037] **Figure 10** shows binding of a target compound and a detectable change in the target compound on binding. A. UV/Vis spectra obtained when silk protein films generated from recombinant honeybee silk protein AmelF3 were bound to increasing NO concentrations. B. Ratio change of absorbance change at 420 nm as NO concentrations are increased. Either the decrease in absorbance at 420 nm or increase in absorbance at 390 nm can be used. The decrease in absorbance at 420 nm can be converted into the fraction of nitric oxide binding site occupied by NO and plotted against NO concentration, and fitted to a sigmoidal curve modified from the Hill equation with a dissociation constant of 6.7 μM and a limit of detection of ~ 1 μM. This data shows compositions of the present invention are capable of binding a target molecule (b), and that target molecule binding results in a detectable change in the composition.

[0038] **Figure 11** shows peroxidase activity of haem-silk materials. A. Shows the spectral changes over time when a haem b-AmelF3 sponge (~1mg) was added to a solution of H_2O_2, aminoantipyrine and phenol. The increase in absorbance at 510 nm is due to the formation of an oxidation product between aminoantipyrine and phenol. The oxidation product is red. B. Peroxidase assay monitoring the change in absorbance at 510 nm with time. As can be seen in the silk film control (without haem; dashed line), peroxidase activity is due to the addition of haem to the film. C. Demonstration that haem b-AmelF3 sponges can be used as recoverable and reusable catalysts. The picture shows a series of wells with 4-aminoantipyrine, phenol and H_2O_2. When a haem-silk sponge is added, the colour changes to red. The sponge was taken out of each well and placed into the well next to it (left to right). The final well is a colourless solution to which the sponge has not yet been added, the second well to the right still has the haem b-AmelF3 sponge.

[0039] **Figure 12** shows that a composition of the present invention can be used to bind ZnPc which has antimicrobial activity. Left; recombinant honeybee silk protein material after leaching in of the photosensitizer zinc phthalocyanine tetrasulfonic acid and extensive washing, showing strong blue colour characteristic of bound zinc

phthalocyanine. Right; recombinant silk film without bound zinc phthalocyanine, washed with 70% MeOH.

[0040] **Figure 13** shows the identity of the metal chelate may be varied, whilst maintaining the features of the invention. Different artificial porphyrins were introduced into honeybee silk films. UV/Vis spectra of honeybee silk films containing artificial porphyrin (copper protoporphyrin IX - CuPPIX), cobalt protoporphyrin IX (CoPPIX) and haem *b* (FePPIX). The ability to vary the metal ion can be used to alter the function of the composition. For example, CoPPIX could be used as nitric oxide sensor with improved sensitivity for nitric oxide and decreased affinity for molecular oxygen.

[0041] **Figure 14** shows an example of the stability of the compositions of the present invention. UV/Vis spectra were measured with freshly prepared (new film) and one year old (1 year old film) composite films comprising recombinant honeybee silk protein AmelF3 and bound haem b, showing that neither the position nor the intensity of the peak has changed, with prolonged storage at ambient room temperatures. Both the new film and one year old film were able to bind NO (dashed line).

[0042] **Figure 15** shows that composite materials of the present invention are unchanged after exposure to a variety of different solvents. A. UV/Vis spectra (untreated: dashed line; treated: solid line) obtained from silk protein films generated from recombinant honeybee silk protein AmelF3 containing bound haem *b* after treatment with various solvents (left: ethyl acetate; middle: chloroform; right: ethanol) showing that the treatment did not significantly affect the position or intensity of the Soret peak. B. Sponges are unchanged after exposure to solvents for 24hrs (no chelate was added to either the silk sponge or the solvent).

[0043] **Figure 16** shows an example of the use of haem-silk materials in electrochemical measurements. Haem b in AmelF3 films were cast onto glassy carbon electrode modified with carbon nanotubes. A clear reversible peak is seen which can be attributed to the Fe3+/Fe2+ redox couples. These redox peaks are not observed in the absence of heme (dotted line) or without AmelF3 silk (dashed-dotted line).

[0044] **Figure 17** shows the use of the haem-silk electrode prepared as described in Example 15 to detect nitric oxide. Upon the addition of nitric oxide a pronounced catalytic current is noted. This catalytic current is attributed to the oxidation of nitric oxide by haem-silk materials can be to determine the nitric oxide concentration in samples.

[0045] **Figure 18** shows the use of the haem-silk electrode prepared as described in Example 15 to detect oxygen. The electrode was held at a constant potential (-300 mV vs Ag/AgCl) under anaerobic conditions (through purging with argon gas). Aliquots of aerated buffer solution we added to introduce oxygen at varying concentrations. The sharp increase in catalytic current is due to the reduction of oxygen by haem-silk materials demonstrating that these materials can be used as an oxygen sensor.

[0046] **Figure 19** shows that an additional haem binding site can be introduced into AmelF3. A coordinating His residue was introduced into AmelF3 with Y76A in the a-position of the coiled-coil. The resulting protein (Y76A A97H) showed a sharp Soret peak at 413 nm indicative of histidine coordination.

[0047] **Figure 20** demonstrates that heme binding in AmelF3 can be increased by addition of a further coordinating residue. A coordinating His residue (His97) was introduced into AmelF3 with Y76H substitution in the a-position of the coiled-coil. Y76H shows a distinct 1:1 ratio of heme binding to silk protein, indicated by the broadening of the Soret peaks a heme ratios above 1:1 and a shift in the position of the peak maxima. No change in both the shape and the position of the Soret peak was noted for Y76H A97H indicating that an extra heme binding site had been introduced and the modified AmelF3 was now able to bind two heme cofactors.

KEY TO THE SEQUENCE LISTING

[0048] SEQ ID NO: 1 – amino acid sequence of *Apis mellifera* (honey bee) silk fibroin 1 (also termed AmelF1 or Xenospira1) with signal sequence

[0049] SEQ ID NO: 2 – amino acid sequence of *Apis mellifera* silk fibroin 2 (also termed AmelF2 or Xenospira2) with signal sequence

[0050] SEQ ID NO: 3 – amino acid sequence of *Apis mellifera* silk fibroin 3 (also termed AmelF3 or Xenospira3) with signal sequence

[0051] SEQ ID NO: 4 – amino acid sequence of *Apis mellifera* silk fibroin 4 (also termed AmelF4 or Xenospira4) with signal sequence

[0052] SEQ ID NO: 5 – nucleotide sequence of *Apis mellifera* silk fibroin 1 (also termed AmelF1 or Xenospira1)

[0053] SEQ ID NO: 6 – nucleotide sequence of *Apis mellifera* silk fibroin 2 (also termed AmelF2 or Xenospira2)

[0054] SEQ ID NO: 7 – nucleotide sequence of *Apis mellifera* silk fibroin 3 (also termed AmelF3 or Xenospira3)

[0055] SEQ ID NO: 8 – nucleotide sequence of *Apis mellifera* silk fibroin 4 (also termed AmelF4 or Xenospira4)

[0056] SEQ ID NO: 9 – amino acid sequence of *Oecophylla smaragdina* (weaver ant) silk fibroin 1 (also termed F1 or GAF1) with signal sequence

[0057] SEQ ID NO: 10 – amino acid sequence of *Oecophylla smaragdina* silk fibroin 2 (also termed F2 or GAF2) with signal sequence

[0058] SEQ ID NO: 11 – amino acid sequence of *Oecophylla smaragdina* silk fibroin 3 (also termed F3 or GAF3) with signal sequence

[0059] SEQ ID NO: 12 – amino acid sequence of *Oecophylla smaragdina* silk fibroin 4 (also termed F4 or GAF4) with signal sequence

[0060] SEQ ID NO: 13 – nucleotide sequence of *Oecophylla smaragdina* silk fibroin 1 (also termed F1 or GAF1)

[0061] SEQ ID NO: 14 – nucleotide sequence of *Oecophylla smaragdina* silk fibroin 2 (also termed F2 or GAF2)

[0062] SEQ ID NO: 15 – nucleotide sequence of *Oecophylla smaragdina* silk fibroin 3 (also termed F3 or GAF3)

[0063] SEQ ID NO: 16 – nucleotide sequence of *Oecophylla smaragdina* silk fibroin 4 (also termed F4 or GAF4)

[0064] SEQ ID NO: 17 – amino acid sequence of *Apis cerana* (Asiatic honey bee) silk fibroin 1

[0065] SEQ ID NO: 18 – amino acid sequence of *Apis cerana* silk fibroin 2

[0066] SEQ ID NO: 19 – amino acid sequence of *Apis cerana* silk fibroin 3

[0067] SEQ ID NO: 20 – amino acid sequence of *Apis cerana* silk fibroin 4

[0068] SEQ ID NO: 21 – amino acid sequence of *Oecophylla smaragdina* silk fibroin 1

[0069] SEQ ID NO: 22 – amino acid sequence of *Oecophylla smaragdina* silk fibroin 2

[0070] SEQ ID NO: 23 – amino acid sequence of *Oecophylla smaragdina* silk fibroin 3

[0071] SEQ ID NO: 24 – amino acid sequence of *Oecophylla smaragdina* silk fibroin 4

[0072] SEQ ID NO: 25 – amino acid sequence of *Polistes dominula* (European paper wasp) silk fibroin 1

[0073] SEQ ID NO: 26 – amino acid sequence of *Polistes dominula* silk fibroin 2

[0074] SEQ ID NO: 27 – amino acid sequence of *Polistes dominula* silk fibroin 3

[0075] SEQ ID NO: 28 – amino acid sequence of *Polistes dominula* silk fibroin 4

[0076] SEQ ID NO: 29 – amino acid sequence of *Apis dorsata* (Giant honeybee) silk fibroin 1

[0077] SEQ ID NO: 30 – amino acid sequence of *Apis dorsata* silk fibroin 2

[0078] SEQ ID NO: 31 – amino acid sequence of *Apis dorsata* silk fibroin 3

[0079] SEQ ID NO: 32 – amino acid sequence of *Apis dorsata* silk fibroin 4

[0080] SEQ ID NO: 33 – amino acid sequence of *Apis florea* (Dwarf honeybee) silk fibroin 1

[0081] SEQ ID NO: 34 – amino acid sequence of *Apis florea* silk fibroin 2

[0082] SEQ ID NO: 35 – amino acid sequence of *Apis florea* silk fibroin 3

[0083] SEQ ID NO: 36 – amino acid sequence of *Apis florea* silk fibroin 4

[0084] SEQ ID NO: 37 – amino acid sequence of *Apis mellifera* silk fibroin 1

[0085] SEQ ID NO: 38 – amino acid sequence of *Apis mellifera* silk fibroin 2

[0086] SEQ ID NO: 39 – amino acid sequence of *Apis mellifera* silk fibroin 3

[0087] SEQ ID NO: 40 – amino acid sequence of *Apis mellifera* silk fibroin 4

[0088] SEQ ID NO: 41 – amino acid sequence of *Bombus impatiens* (common eastern bumblebee) silk fibroin 2

[0089] SEQ ID NO: 42 – amino acid sequence of *Bombus terrestris* (buff tailed bumblebee) silk fibroin 1

[0090] SEQ ID NO: 43 – amino acid sequence of *Bombus terrestris* silk fibroin 2

[0091] SEQ ID NO: 44 – amino acid sequence of *Bombus terrestris* silk fibroin 3

[0092] SEQ ID NO: 45 – amino acid sequence of *Bombus terrestris* silk fibroin 4

[0093] SEQ ID NO: 46 – amino acid sequence of *Camponotus floridanus* (florida carpenter ant) silk fibroin 2

[0094] SEQ ID NO: 47 – amino acid sequence of *Camponotus floridanus* silk fibroin 3

[0095] SEQ ID NO: 48 – amino acid sequence of *Camponotus floridanus* silk fibroin 4

[0096] SEQ ID NO: 49 – amino acid sequence of *Harpegnathos saltator* (indian jumping and or Jerdon's jumping ant) silk fibroin 1

[0097] SEQ ID NO: 50 – amino acid sequence of *Harpegnathos saltator* silk fibroin 2

[0098] SEQ ID NO: 51 – amino acid sequence of *Harpegnathos saltator* silk fibroin 3

[0099] SEQ ID NO: 52 – amino acid sequence of *Harpegnathos saltator* silk fibroin 4

[00100] SEQ ID NO: 53 – amino acid sequence of *Myrmecia forficate* (bulldog ant) silk fibroin 1

[00101] SEQ ID NO: 54 – amino acid sequence of *Myrmecia forficate* silk fibroin 2

[00102] SEQ ID NO: 55 – amino acid sequence of *Myrmecia forficate* silk fibroin 3

[00103] SEQ ID NO: 56 – amino acid sequence of *Myrmecia forficate* silk fibroin 4

[00104] SEQ ID NO: 57 – amino acid sequence of *Megachile rotundata* (alfalfa leafcutter bee) silk fibroin 2

[00105] SEQ ID NO: 58 – amino acid sequence of *Megachile rotundata* silk fibroin 3

[00106] SEQ ID NO: 59 – amino acid sequence of *Megachile rotundata* silk fibroin 4

[00107] SEQ ID NO: 60 – amino acid sequence of *Osmia cornuta* (builder bee) silk fibroin 2

[00108] SEQ ID NO: 61 – amino acid sequence of *Osmia cornuta* silk fibroin 4

[00109] SEQ ID NO: 62 – amino acid sequence of *Vespa simillima xanthoptera* (Japanese yellow hornet) silk fibroin 1

[00110] SEQ ID NO: 63 – amino acid sequence of *Vespa simillima xanthoptera* silk fibroin 2

[00111] SEQ ID NO: 64 – amino acid sequence of *Vespa simillima xanthoptera* silk fibroin 3

[00112] SEQ ID NO: 65 – amino acid sequence of *Vespa simillima xanthoptera* silk fibroin 4

[00113] SEQ ID NO: 66 – amino acid sequence of *Vespa analis* (yellow-vented hornet) silk fibroin 1

[00114] SEQ ID NO: 67 – amino acid sequence of *Vespa analis* silk fibroin 2

[00115] SEQ ID NO: 68 – amino acid sequence of *Vespa analis* silk fibroin 3

[00116] SEQ ID NO: 69 – amino acid sequence of *Vespa analis* silk fibroin 4

[00117] SEQ ID NO: 70 – amino acid sequence of *Vespa mandarinia* (Asian giant hornet) silk fibroin 1

[00118] SEQ ID NO: 71 – amino acid sequence of *Vespa mandarinia* silk fibroin 2

[00119] SEQ ID NO: 72 – amino acid sequence of *Vespa mandarinia* silk fibroin 3

[00120] SEQ ID NO: 73 – amino acid sequence of *Vespa mandarinia* silk fibroin 4

[00121] SEQ ID NO: 74 – amino acid sequence of *Tenodera australasiae* protein Mantis Fibroin 1

[00122] SEQ ID NO: 75 – amino acid sequence of *Tenodera australasiae* protein Mantis Fibroin 2

[00123] SEQ ID NO: 76 – amino acid sequence of *Archimantis monstrosa* silk fibroin 1

[00124] SEQ ID NO: 77 – amino acid sequence of *Archimantis monstrosa* silk fibroin 2

[00125] SEQ ID NO: 78 – amino acid sequence of *Pseudomantis albofimbriata* silk fibroin 1

[00126] SEQ ID NO: 79 – amino acid sequence of *Pseudomantis albofimbriata* silk fibroin 2

[00127] SEQ ID NO's: 80 to 82 – Oligonucleotide primers.

DETAILED DESCRIPTION

[00128] The present invention is based in part on the characterisation that polypeptides having a coiled coil structure are able to bind a chelate comprising a chelating agent and a metal ion. For example, the present inventors have demonstrated a silk polypeptide having a coiled coil structure is able to bind a chelate and co-ordinate the metal ion of the chelate. Furthermore, the present inventors have demonstrated that the co-ordination of the metal ion of the chelate can causes a shift in the location of a peak in the UV-visible spectrum (e.g. the Soret peak). The present inventors have also shown that the binding of a molecule able to bind the chelate bound to the polypeptide causes a detectable change, and this can be used as the basis of a sensor, for example a biosensor, that may be used for detecting a molecule of interest.

[00129] Accordingly, in a first aspect, the present invention provides a composition comprising: a polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; and a chelate comprising a chelating agent and a metal ion; and

wherein the chelate is bound to at least one amino acid of the polypeptide. In some embodiments, the chelating agent is bound to at least one amino acid of the polypeptide. In some embodiments, the metal ion is bonded to at least one amino acid of the polypeptide by a co-ordinate bond. In some embodiments, the metal ion is bonded to at least one amino acid of the polypeptide by a co-ordinate bond and the chelating agent is bound to at least one amino acid of the polypeptide.

[00130] Advantageously, the compositions of the present invention may be processed to form a material, preferably a water insoluble material, and are highly stable at room temperature for extended periods of time. In some embodiments, the compositions of the present invention and materials formed from the compositions of the present invention are able to reversibly bind a molecule of interest. Importantly, the compositions of the present invention, and materials formed from the compositions retain the ability to bind and/or detect the molecule of interest over extended periods.

Polypeptides

[00131] The term "polypeptide", as used herein, includes amino acid polymers of any length. The protein may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labelling component. Also included are, for example, proteins containing one or more analogs of an amino acid (including, for example, unnatural or non-natural amino acids, etc.), as well as other modifications known in the art. Proteins can occur as single chains or associated chains. Associated chains may be joined by non-covalent or covalent interactions. In an embodiment, the polypeptide is a chain of naturally occurring amino acids.

[00132] Polypeptides useful for the invention can be prepared by various means (e.g. isolation and purification from source, recombinant expression, purification from cell culture, chemical synthesis, etc.) and in various forms (e.g. native, fusions, non-glycosylated, lipidated, etc.). They are preferably prepared in substantially pure form

(i.e. substantially free from host cell proteins). Typically, the polypeptide is substantially pure when it is at least 60%, by weight, of total protein present. For example, the preparation is at least 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, more preferably at least 90%, by weight, of total protein present. In an embodiment, the polypeptide is produced by recombinant means, such as expression in a suitable host cell such as a yeast cell or bacterial cell.

[00133] As used herein, "portion" is understood to refer to a portion of a polypeptide which maintains a defined characteristic or activity of the full-length polypeptide. For example, having the ability to form a coiled coil structure and/or having the ability to reproduce any one or more characteristics of a silk protein.

[00134] As disclosed herein, at least a portion of the polypeptide may form a coiled coil structure. A coiled coil structure comprises at least two alpha helices that coil together like the strands of a rope. A coiled coil structure may comprise between two and seven alpha helices, such as two, three, four, five, six or seven alpha helices. In some embodiments, the coiled coil structure comprises three, four or five alpha helices. Coiled coil structures may form from intra-chain or inter-chain interactions.

[00135] In some embodiments, the portion of the polypeptide that has a coiled coil structure comprises at least 35 amino acids, at least 42 amino acids, at least 49 amino acids, at least 56 amino acids, at least 63 amino acids, at least 70 amino acids, at least 77 amino acids, at least 84 amino acids, at least 91 amino acids, at least 98 amino acids, at least 105 amino acids, at least 112 amino acids, at least 119 amino acids, at least 126 amino acids, at least 133 amino acids, at least 140 amino acids, at least 147 amino acids, at least 154 amino acids, at least 161 amino acids, at least 168 amino acids, at least 175 amino acids, at least 182 amino acids, at least 189 amino acids, at least 196 amino acids, at least 203 amino acids, at least 210 amino acids or at least 217 amino acids. In an embodiment, the portion of the polypeptide that has a coiled coil structure comprises about 35 to about 500 amino acids.

[00136] In some embodiments, at least 16% of the amino acids in the coiled coil structure are alanine residues. For example, at least 18%, at least 20%, at least 22%,

at least 24%, at least 26%, at least 28% or at least 30% of the amino acids in the coiled coil structure are alanine residues.

[00137] Typically, alpha-helices contain about 3.6 amino acid residues per helical turn. In the case of alpha-helices capable of forming a coiled coil structure, hydrophobic and hydrophilic amino acid residues are spaced along the linear sequence of the peptide such that when the polypeptide or portion of a polypeptide assumes an alpha-helical conformation, the hydrophobic and hydrophilic amino acid residues are respectively segregated to separate faces of the helix, forming an amphipathic structure. The segregation of hydrophobic and hydrophilic amino acid residues in an alpha-helix can be visualized in a helical wheel. Certain hydrophobic and hydrophilic amino acid residues are preferred in constructing alpha-helical peptides capable of forming a coiled coil structure. Naturally occurring hydrophobic amino acid residues are Leu, Ala, Ile, Val and Phe. Preferred naturally occurring hydrophilic amino acid residues are Ser, Glu, Lys, Gln and Asp. As will be understood by a person skilled in the art the polypeptide sequence capable of forming a coiled structure can vary. A large number of combinations and permutations of different amino acids in the polypeptide sequence can achieve the effect of producing an amphipathic alpha helix, which allows the formation of a coiled coil in association with another polypeptide. For example, in some embodiments serine is common in the core of coiled coils formed by the polypeptides of the present invention but not in other coiled coils.

[00138] Polypeptides capable of forming a coiled coil structure usually comprise repeats of the heptad sequence abcdefg. In one embodiment the polypeptide comprises a portion comprising at least 5 copies of the heptad sequence abcdefg. Polypeptides useful for the invention may comprise any number of heptad repeats greater than 4. For example, the polypeptides may comprise a portion having a coiled coil structure comprising at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39 at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50, or more copies of the heptad

sequence abcdefg. In one particular example, the polypeptides comprise a portion having a coiled coil structure comprising at least 19, or at least 23 copies of the heptad sequence abcdefg.

[00139] In one embodiment, the polypeptides useful for the invention comprise about 9 to about 30, such as about 15 to about 25, or about 19 to about 23 heptad repeats, or about 19 to about 23 repeats. In some embodiments, the polypeptides comprise a portion having a coiled coil structure comprising between 22 and 28 repeats.

[00140] The heptad repeats may be contiguous in the polypeptide sequence or may not be contiguous in the polypeptide sequence. Thus, the polypeptide may comprise any number of contiguous heptad sequences, provided that the total number of heptad sequences in the polypeptide is at least 5. Preferably, the polypeptide comprises at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25 or more contiguous heptad sequences. In one example, the polypeptide comprises at least 19, such as at least 20, at least 21, at least 22, or at least 23 contiguous heptad sequences. In another example, the polypeptide comprises about 10 to about 30, such as about 15 to about 25, or about 19 to about 23 contiguous heptad repeats. Preferably, the polypeptides comprise about 19 or about 23 contiguous heptad repeats.

[00141] The heptad repeats may comprise any portion of the polypeptide useful for the invention. For example, the heptad repeats may comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, of the length of the polypeptide, or the entire polypeptide.

[00142] In another example, the heptad repeats may comprise about 60% to about 90%, such as about 70% to about 80% of the polypeptide. Thus, about 60% to about 90%, such as about 70% to about 80% of the polypeptide may comprise a coiled coil structure.

[00143] In the classic coiled coil, the amino acids at the a and d position are hydrophobic residues, such as but not limited to alanine, isoleucine, leucine or valine. These amino acids tend to be more hydrophobic on average than amino acids at other positions in the heptad sequence. This is thought to stabilise the formation of the coiled coil via hydrophobic and van der Waals interactions. Hydrophobicity of an amino acid residue can be determined by any method known in the art. For example, hydrophobicity can be predicted based on the physiochemical properties of the amino acid side chains, or may be determined by partitioning of an amino acid between two immiscible liquid phases. The use of these methods to determine the relative hydrophobicity of each of the naturally occurring amino acids has resulted in the production of several known hydrophobicity scales (see, by way of example only, Kallol et al., 2003; Kyte and Doolittle, 1982; Eisenberg, 1984; Rose and Wolfenden, 1993). Any of these, or other known hydrophobicity scales, can be used to determine the hydrophobicity (and hence, the average hydrophobicity) of the amino acids present at each position in the heptad sequence abcdefg. In one example, amino acid hydrophobicity is determined according to the Eisenberg scale. In a particular example, the average hydrophobicity of amino acids at positions a and/or d in the heptad sequence is positive according to the Eisenberg scale, and the average hydrophobicity of amino acids at each of the remaining positions in the heptad sequence is negative according to the Eisenberg scale. Generally, the following amino acids are considered to be more hydrophobic than others: cysteine, glycine, isoleucine, leucine, methionine and valine. Aromatic amino acids are also generally considered to be more hydrophobic than non-aromatic amino acids.

[00144] The amino acids at the e and g positions may be charged, for example but not limited to glutamate or lysine. This is thought to facilitate the formation of interhelical electrostatic interactions that stabilise the coiled coil structure. Amino acids at the b, c and f positions tend to be hydrophilic as these amino acids are often exposed to solvent. However, the above is only a guide and the person skilled in the art would be aware that variations may occur at any of the a, b, c, d, e, f and g positions to facilitate specificity, novel functions, oligomerisation and the like.

[00145] In one embodiment of the present invention, the amino acids at positions a and/or d in the heptad sequence are selected from alanine, serine, isoleucine, leucine or valine, preferably serine or alanine.

[00146] In an embodiment, at least 15% of the amino acids at position a in the heptad repeats in the polypeptides useful for the invention are alanine residues. For example, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or more of the amino acids at position a may be alanine residues. Preferably at least 45% of the amino acids at position a in the heptad repeats in the polypeptides useful for the invention are alanine residues.

[00147] In another example, from about 30% to about 70%, such as from about 40% to about 60%, or from about 45% to about 55% of the amino acids at position a in the heptad repeats in the polypeptides useful for the invention are alanine residues. In one example, about 50% of the amino acids at position a in the heptad repeats in the polypeptides useful for the invention are alanine residues.

[00148] The relative proportions of alanine at position a and d in the heptad repeats can vary, provided that at least 15% of the amino acids at position a and d are alanine residues Thus, the polypeptides useful for the invention can comprise heptad repeats wherein at least 25% of the amino acids at position a are alanine residues.

[00149] The composition of amino acids making up each heptad repeat may be the same or may differ from one heptad sequence to another. As will be understood by a person skilled in the art, a large number of combinations and permutations of different amino acids in the heptad sequence abcdefg can achieve the same effect of producing a coiled coil structure, which allows the formation of a coiled coil in association with another polypeptide. Guidance regarding amino acid substitutions which can be made to the polypeptides disclosed herein is provided, by way of example only, in Table 1. Where a predicted useful amino acid substitution based on the experimental data provided herein is in any way in conflict with the exemplary substitutions provided in Table 1 it is preferred that a substitution based on the experimental data is used.

[00150] In addition, the polypeptides may comprise certain disruptions within and/or between each heptad repeat which nevertheless allow the formation of a coiled coil structure. For example, a heptad sequence may be truncated by one or more amino

acids or extended by one or more amino acids, whilst still forming a coiled coil structure. Thus, as stated above, two copies of the heptad sequence abcdefg may be separated by one or more amino acids, which nevertheless still allows the formation of an coiled coil structure.

[00151] Any portion of the polypeptide may comprise a coiled coil structure. For example, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or the entire polypeptide may comprise a coiled coil structure. In one example, at least 70% of the polypeptide comprises a coiled coil structure.

[00152] The remainder of the polypeptide that does not form a coiled coil structure can form any secondary protein structure or may not be structured. For example, the remainder of the polypeptide may form random coils, alpha helices, beta strands, and/or beta-sheets and the like.

[00153] In some embodiments, at least a portion of the polypeptide may form or be capable of forming a beta sheet. A beta sheet comprises beta strands connected by backbone hydrogen bonds. A beta sheet may comprise at least two beta strands. For example, in a material described herein, at least a portion of the polypeptide may form a beta sheet. Beta sheets may form from intra-chain or inter-chain interactions between beta strands.

[00154] The present inventors have demonstrated that silk polypeptides can bind to a chelate comprising a chelating agent and a metal ion. Accordingly, in one aspect the polypeptide is a silk polypeptide. In some embodiments, the polypeptides are silk proteins (including recombinant silk proteins) from, or are a mutant thereof, aculeate Hymenoptera. Examples of Hymenopterans include, but are not limited to, any species of the Suborder Apocrita (bees, ants and wasps), which include the following Families of insects; Chrysididae (cuckoo wasps), Formicidae (ants), Mutillidae (velvet ants), Pompilidae (spider wasps), Scoliidae, Vespidae (paper wasps, potter wasps, hornets), Agaonidae (fig wasps), Chalcididae (chalcidids), Eucharitidae (eucharitids), Eupelmidae (eupelmids), Pteromalidae (pteromalids), Evaniidae (ensign wasps), Braconidae, Ichneumonidae (ichneumons), Megachilidae, Apidae, Colletidae,

Halictidae, and Melittidae (oil collecting bees). For example, the Hymenoptera may be, but not limited to, *Apis mellifera* (common names include honeybee), *Apis dorsata*, *Apis florea*, *Oecophylla smaragdina* (common names include weaver ant and green ant), *Polistes dominula*, *Megachile rotundata, Myrmecia foricata, Camponotus floridanus, Harpegnathos saltator*, *Osmia cornuta*, *Vespa simillima xanthoptera*, *Vespa analis*, *Vespa mandarina*, *Bombus impatiens* or *Bombus terrestris*. In some embodiments, the polypeptides are silk proteins (including recombinant silk proteins) from, or are a mutant thereof, Dictyoptera. For example, the Dictyoptera may be, but not limited to, *Pseudomantis albofimbriata*, *Tenodera australasiae* or *Archimantis monstrosa*. In some embodiments, the polypeptides are silk proteins (including recombinant silk proteins) from, or are a mutant thereof, Neuroptera. Examples of Neuropterans include species from the following insect Families: Mantispidae (see Walker et al., 2012), Chrysopidae (lacewings), Myrmeleontidae (antlions), and Ascalaphidae (owlflies). For example, the Neuroptera may be, but not limited to, *Mallada signata*. Examples of such proteins are described in WO 2007/038837 and WO 2013/142901. These silk polypeptides have the advantage that they can be readily expressed in high levels using fermentation (at least 1 g/litre).

[00155] In a preferred embodiment, a polypeptide useful for the invention can be purified from, or is a mutant of a polypeptide purified from, a species of Hymenoptera or Neuroptera. Preferably, the species of Hymenoptera is *Apis mellifera*.

[00156] In some embodiments, a polypeptide useful for the invention can be purified from, or is a mutant of a polypeptide purified from, a species of Oecophylla. Preferably, the species of Oecophylla is *Oecophylla smaragdina*.

[00157] The polypeptides useful for the invention are exemplified by a number of particular proteins whose sequences are provided in the following SEQ ID NOs. For example, the polypeptides useful for the invention include, but are not limited to, *Apis mellifera* (honey bee) silk fibroin 1 (also termed AmelF1 or Xenospira1) (SEQ ID NO:1), *A. mellifera* silk fibroin 2 (also termed AmelF2 or Xenospira2) (SEQ ID NO:2), *A. mellifera* silk fibroin 3 (also termed AmelF3 or Xenospira3) (SEQ ID NO:3), *A. mellifera* silk fibroin 4 (also termed AmelF4 or Xenospira4) (SEQ ID NO:4), *Oecophylla smaragdina* (weaver ant) silk fibroin 1 (also termed F1, GA1 or GAF1) (SEQ ID NO:9), *O. smaragdina* silk fibroin 2 (also termed F2, GA2 or GAF2) (SEQ ID

NO:10), *O. smaragdina* silk fibroin 3 (also termed F3, GA3 or GAF3) (SEQ ID NO:11) and *O. smaragdina* silk fibroin 4 (also termed F4, GA4 or GAF4) (SEQ ID NO:12), as well as those described in WO2013142901 A.

[00158] In a preferred embodiment, the polypeptide is AmelF3.

[00159] In another embodiment, the polypeptide is GA1 or GA3.

[00160] As used herein, the terms "silk protein" and "silk polypeptide" are used interchangeably and refer to a fibrous protein/polypeptide that can be used to produce materials such as silk fibre, silk film, silk sponges, silk particles and/or a fibrous protein complex. Typically, the silk proteins will be produced by recombinant expression. However, the silk proteins can be purified from a natural source or produced artificially such as, for example, by solid phase peptide synthesis or the like. Silk proteins may have a sequence corresponding to a naturally occurring silk protein or be a man-made variant thereof. Such variants not only include small substitutions, deletions and additions, but also encompass significant rearrangement of the native sequences where, for example, heptads are reordered so they bear no resemblance to the primary amino acid sequence of the native protein but because of the heptad structure are still functional silk proteins.

[00161] As discussed above, the present inventors have demonstrated at least one amino acid of a polypeptide as described herein is able to bind to a chelate. In some embodiments, a polypeptide as described herein is able to bind to at least one chelate as described herein. In other embodiments, a polypeptide described herein is able to bind to two or more chelates described herein.

[00162] Accordingly, a polypeptide as described herein comprises at least one chelate binding site. For example, when the chelate is haem, the chelator binding site is a haem binding site. In some embodiments, a polypeptide as described herein comprises two or more chelate binding sites.

[00163] In some embodiments, a polypeptide as described herein is able to bind to at least one chelating agent as described herein. In other embodiments, a polypeptide described herein is able to bind to two or more chelating agents described herein. In some embodiments, the polypeptide useful for the invention comprises at least one

charged residue which can bind to the chelating agent. Charged residues comprise but are not limited to arginine, lysine, glutamate and aspartate. In some embodiments, the at least one amino acid residue bound to the chelating agent is a positively charged amino acid such as arginine or lysine. In some embodiments, the at least one amino acid residue bound to the chelating agent is arginine. In some embodiments, the at least one amino acid residue bound to the chelating agent is a charged residue located up to 12 Å from the at least one amino acid residue bound to the metal ion by a co-ordinate bond.

[00164] Furthermore, the present inventors have demonstrated that in some embodiments the metal ion is bound to at least one amino acid of the polypeptide by a co-ordinate bond. As used herein the term "coordinate bond" refers to a kind of 2-center, 2-electron covalent bond in which the two electrons derive from the same atom. A coordinate bond can also be referred to as a dipolar bond or a dative covalent bond.

[00165] In an embodiment, the core of the coiled coil portion comprises at least one, or two, or three, or four or more, coordinating amino acid residues at amino acid position a and/or d of the heptads in the core. The "core" of coiled coil proteins is well known in the art (Lupas and Gruber, 2005). Coiled coil regions form alpha helixes, and two or more such helixes coil around each other with the hydrophobic residues inside and forming a long thin hydrophobic core. Thus, the coiled coil core is more hydrophobic than the outside of the core.

[00166] In one embodiment, the polypeptide includes a single coordinating amino acid residue. In one embodiment, the polypeptide includes two coordinating amino acid residues. In one embodiment, the polypeptide includes three coordinating amino acid residues. In one embodiment, the polypeptide includes four coordinating amino acid residues. In some embodiments, the coordinating amino acid is selected from the group consisting of histidine, cysteine, methionine, tyrosine, lysine or tryptophan.

[00167] An amino acid residue that is present in a polypeptide and which residue provides a coordinating contact with a metal ion-chelator complex is referred to herein as a coordinating amino acid or coordinating residue. Amino acids suitable for use as a coordinating amino acid in a polypeptide include naturally-occurring amino acids

known in the art to provide a ligand for metal cations in metalloproteins, and include His, Cys, Met, Lys, Trp, Glu and Tyr. Amino acids suitable for use as a coordinating amino acid residue in a polypeptide also include non-naturally-occurring amino acids known in the art to provide a ligand for metal cations. Such non-naturally occurring amino acids may include, but are not limited to, (2,2-bipyridin-5yl)alanine (Bpy-Ala), (8-hydroxyquinolin-3-yl)alanine, 2-amino-3-[4-hydroxy-3-(1H-pyrazol-1-yl)phenyl] propanoic acid (pyTyr) and 2-amino-3-(8-hydroxyquinolin-5-yl)propanoic acid (HqAla).

[00168] For example, the present inventors demonstrate that when a solution of haem *b* (which is found in haemoglobin and cytochromes P450) in aqueous methanol was added to a honeybee silk sponge, the greyish green haem *b* solution was immediately absorbed into the sponge and within seconds the colour of the sponge changed to red. This red colouration in honeybee silk remained after exhaustive washing of the silk-haem material. The colour change observed with honeybee silk indicates a change in the coordination of the iron metal centre within the haem group, producing a material with a similar coordination to red haemoglobin. Furthermore, the haem group remained bound within the silk protein matrix and could not be washed out, indicating that the haem group was held within the protein matrix. Somewhat unexpectedly, without any modification, naturally honeybee silk was able to bind and coordinate haem cofactors in a manner resembling naturally occurring haem proteins.

[00169] To test the coordination of the iron centre, transparent honeybee silk films were prepared to monitor the spectral properties of the material using UV/Vis spectroscopy. Haem *b* was introduced either through co-drying a solution of silk protein and haem *b,* or by “leaching” haem *b* into a preformed film by soaking the film in solutions of haem *b* overnight. Haem proteins have a characteristic Soret peak in their UV/Vis spectrum at ~ 400 nm which is extremely sensitive to changes in the coordination of the iron haem atom. In the case of iron porphyrins such as haem *b*, broad Soret peaks below 400 nm indicate a 4 coordinate iron centre with the iron only coordinated to the porphyrin ring. When the haem group is coordinated to an amino acid (5 coordinate iron centre), the Soret band red shifts to above 400 nm and sharpens. The ultraviolet-visible spectrum from honeybee silk-haem *b* films show a sharp Soret peak at 410 nm confirming that that iron haem centre is coordinated to the honeybee silk protein.

[00170] Accordingly, the present invention also provides a composition comprising: a polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; and a chelate comprising a chelating agent and a metal ion; and wherein the chelate is bonded to at least one amino acid of the polypeptide, and wherein the metal ion is bonded to the at least one amino acid of the polypeptide by a co-ordinate bond.

[00171] The coordination of haem *b* to unmodified honeybee silk protein was unexpected, given that the silk protein does not contain any of the typical coordinating residues such as histidine, cysteine of methionine. To determine the nature of the coordinating amino residue the present inventors investigated the stoichiometry of haem binding through varying the amount of haem *b* added to the silk protein and using Raman spectroscopy. At low haem loadings (haem:protein molar ratios of 1:1 and 1:2), a sharp Soret peak at 410 nm was observed, indicative of all the haem being coordinated. As the concentration of haem *b* relative to the protein was increased, the Soret peak broadened and shifted to lower wavelengths indicating an increase in the amount of uncoordinated haem. The finding that all haem was coordinated at 1:1 haem:silk ratio suggested that a single amino acid within each silk monomer was responsible for coordination.

[00172] The identity of the coordinating amino acid of the polypeptide was investigated using Raman spectroscopy. Raman spectroscopy measures stretching frequencies between the iron centre and the coordinating ligand, these stretching frequencies are indicative of the nature of ligand. The Raman spectrum of the silk-haem film excited at 785 nm excitation showed a broad peak centred at 594 cm^{-1}, which was specific to the silk-haem film. Haem proteins which have a tyrosine coordinating ligand show similar Fe-Tyr stretches. Mature recombinant honeybee silk protein 3 (SEQ ID NO:39) contains a single tyrosine residue (Tyr76) located in the core of the predicted coiled coil. The Raman spectrum indicated that Tyr76 was the most likely candidate coordinating to the haem centre.

[00173] To test whether Tyr76 was indeed the coordinating ligand, Tyr76 was replaced with an alanine using site directed mutagenesis of the silk gene. The UV/Vis spectrum of the Tyr76Ala substituted protein had a broad Soret peak at 395 nm indicating that the coordination noted in unmodified honeybee silk had been reversed through this single amino acid substitution. When haem *b* was added to sponges

prepared using Tyr76Ala silk protein, no pronounced colour change was observed, however the green haem *b* colour does not wash out with aqueous methanol indicating that the haem *b* cofactor is bound to the silk protein through hydrogen bonding, but not coordinated to the silk. Accordingly, bonding of a metal ion to at least one amino acid of the polypeptide is a preferred feature of the present invention.

[00174] When the at least one polypeptide comprises an AmelF3 polypeptide, the present inventors have demonstrated that, the tyrosine at position 76 of AmelF3 (SEQ ID NO: 39) forms a co-ordinate bond with the metal ion of the chelate.

[00175] Amino acids surrounding the coordinating amino acid in space may play a role in coordinating with the metal ion of the chelate. In some embodiments, a neighboring polar or charged residue may play a role in polarizing and/or activating the coordinating residue so that it may coordinate the metal ion. For example, the inventors have demonstrated that mutating Ser80 of AmelF3 to an alanine does not impact binding of the polypeptide to the chelating agent but prevents coordination of the metal ion. As another example, the inventors have demonstrated that Ser80 of AmelF3 plays a role in the coordination of the metal ion. Without wishing to be bound by theory it is thought that the serine polarises the coordinating residue allowing coordinate binding to the metal ion.

[00176] In some embodiments, the coordinating amino acid residue is located 20 Å or less, 18 Å or less, 16 Å or less 14 Å or less, 12 Å or less, 10 Å or less or 8 Å or less from at least one charged residue, for example, Asp, Glu, Lys or Arg. In some embodiments, the coordinating amino acid residue is located 20 Å or less, 18 Å or less, 16 Å or less 14 Å or less, 12 Å or less, 10 Å or less or 8 Å or less from at least one polar residue, for example, Ser, Thr, Gln, Asn, His, Tyr, Cys, Met or Trp.

[00177] In one embodiment a metal ion binding site and/or chelating agent binding site is engineered into a polypeptide. For example, random mutagenesis or site directed mutagenesis is performed to engineer the protein such that it contains the necessary residues to enable chelate binding to the protein and/or coordination of a metal ion. Therefore the DNA sequence which encodes the polypeptide of this invention, either isolated or incorporated into a vector can be used to produce a polypeptide useful for the invention. This sequence is then expressed in, and the

polypeptide purified from, a cell. (Alternatively it is possible that the polypeptide can be produced using a solid phase peptide synthesis). The resulting polypeptide, which is capable of binding a chelate, is then incubated with an excess of that chelate to ensure binding to the polypeptide.

[00178] In one example, the Ala residue at position 97 of mature AmelF3 (SEQ ID NO: 39) is mutated to a coordinating amino acid residue such as His, Cys, Met, Lys, Trp, Glu or Tyr. In one particular example, the Ala residue at position 97 of AmelF3 is mutated to His.

[00179] In another example, the Tyr residue at position 76 of the mature form of AmelF3 (SEQ ID NO: 39) is mutated to His.

[00180] Metal ions are found in one-third of all proteins and play important structural and functional roles. Significant effort has been directed towards understanding the role of the polypeptide in tuning the metal ion properties. A goal of *de novo* synthesis is to utilise design principles so as to generate functional artificial metalloproteins. Much research has focussed on the mutagenesis studies of native protein scaffolds, or re-engineering of metal ion sites into other protein scaffolds, however this work has been hampered by the complexity of natural scaffolds. Accordingly, in another embodiment, forming a composition of the present invention involves the *de novo* (from scratch) design of a polypeptide able to bind a chelator and/or bond to a metal ion.

[00181] In one embodiment, the polypeptides useful for the invention may have a size ranging from between about 29 kDa to about 45 kDa. For example, the polypeptides may have a size of about 33 kDa.

[00182] In one embodiment, the polypeptide of the present invention is a derived from a native polypeptide. For example, a native polypeptide may be modified by incorporating natural or non-natural amino acids (herein, the terms unnatural and non-natural amino acids are used interchangeably) to enhance or modify binding of a chelator and/or a bonding to a metal ion.

[00183] As will be appreciated from the present disclosure, the exact amino acid sequence of the polypeptides (and hence, the exact nucleic acid sequence of the

polynucleotides) can vary whilst still providing a polypeptide having a structure that is capable of forming a coiled coil in association with itself or another polypeptide. The exemplified sequences should therefore be considered as examples only, and it will be appreciated that significant variation from these particular sequences may be tolerable.

[00184] In a particular example, the polypeptide comprises an amino acid sequence selected from:

i) an amino acid sequence as provided in any one of SEQ ID NO:1 to SEQ ID NO:4 or SEQ ID NO:9 to SEQ ID NO:12; and

ii) an amino acid sequence which is at least 25% identical to any one or more of SEQ ID NO:1 to SEQ ID NO:4 or SEQ ID NO:9 to SEQ ID NO:12; and

iii) a biologically active fragment of i) or ii).

[00185] In another particular example, the polypeptide comprises an amino acid sequence selected from:

i) an amino acid sequence as provided in any one of SEQ ID NO:1 to SEQ ID NO:4 or SEQ ID NO:9 to SEQ ID NO:12 or SEQ ID NO:17 to SEQ ID NO:79; and

ii) an amino acid sequence which is at least 25% identical to any one or more of SEQ ID NO:1 to SEQ ID NO:4 or SEQ ID NO:9 to SEQ ID NO:12 or SEQ ID NO:17 to SEQ ID NO:79; and

iii) a biologically active fragment of i) or ii).

[00186] In another particular example, the polypeptide comprises an amino acid sequence selected from:

i) an amino acid sequence as provided in any one of SEQ ID NO:17 to SEQ ID NO:79; and

ii) an amino acid sequence which is at least 25% identical to any one or more of SEQ ID NO:17 to SEQ ID NO:79; and

iii) a biologically active fragment of i) or ii).

[00187] The % identity of a polypeptide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. The query sequence is at least 50 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 50 amino acids. More preferably, the query sequence is at least 100 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 100 amino acids. Even more preferably, the query sequence is at least 250 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 250 amino acids. Even more preferably, the GAP analysis aligns the two sequences over their entire length.

[00188] With regard to a polypeptide, it will be appreciated that % identity figures higher than those provided herein will encompass preferred embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that the polypeptide or polynucleotide comprises an amino acid sequence which is at least 25%, more preferably at least 35%, more preferably at least 40%, more preferably at least 45%, more preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

[00189] Amino acid sequence mutants of the polypeptides useful for the invention can be prepared by introducing appropriate nucleotide changes into a nucleic acid, or by *in vitro* synthesis of the desired polypeptide. Such mutants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final

construct, provided that the final polypeptide product possesses the desired characteristics.

[00190] Mutant (altered) polypeptides can be prepared using any technique known in the art. For example, a polynucleotide disclosed herein can be subjected to in vitro mutagenesis. Such in vitro mutagenesis techniques include sub-cloning the polynucleotide into a suitable vector, transforming the vector into a "mutator" strain such as the *E. coli* XL-1 red (Stratagene) and propagating the transformed bacteria for a suitable number of generations. In another example, the polynucleotides are subjected to DNA shuffling techniques as broadly described by Harayama (1998). These DNA shuffling techniques may include genes encoding polypeptides described herein and possibly also genes related to those described herein. Products derived from mutated/altered DNA can readily be screened using techniques described herein to determine if they can be used as silk proteins.

[00191] In designing amino acid sequence mutants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting other residues adjacent to the located site.

[00192] Amino acid sequence deletions may be any length but generally range from about 1 to 15 residues, preferably about 1 to 10 residues or about 1 to 7 residues and typically about 1 to 7 contiguous residues.

[00193] Substitution mutants have at least one amino acid residue in the polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as important for function. Other sites of interest are those in which particular residues obtained from various strains or species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are preferably substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "exemplary substitutions".

Table 1. Exemplary substitutions

Original Residue	**Exemplary Substitutions**
Ala (A)	val; leu; ile; gly; cys; ser; thr
Arg (R)	lys
Asn (N)	gln; his
Asp (D)	glu
Cys (C)	ser; thr; ala; gly; val
Gln (Q)	asn; his
Glu (E)	asp
Gly (G)	pro; ala; ser; val; thr
His (H)	asn; gln
Ile (I)	leu; val; ala; met
Leu (L)	ile; val; met; ala; phe
Lys (K)	arg
Met (M)	leu; phe; ile
Phe (F)	leu; val; ala
Pro (P)	gly
Ser (S)	thr; ala; gly; val; gln; cys
Thr (T)	ser; gln; ala; cys
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu; met; phe; ala; ser; thr; cys
A non-cordinating residue (for example Ala)	A cordinating residue (his, cys, met, tyr, lys, glu or trp)
A cordinating residue	A different cordinating residue (selected from his, cys, met, tyr, lys, glu or trp where relevant)

[00194] As used herein, a "biologically active fragment" of a polypeptide may be capable of forming or may form a semi-crystalline material (that is, a material with regions of ordered molecular structure (crystallites) within an amorphous matrix). Alternatively or in addition, the biologically active fragment may be capable of forming or may form filamentous molecules. Thus, the biologically active fragment may be

capable of being used to produce a silk fibre, silk film, silk powder, silk sponge, silk mat and the like. Biologically active fragments can be any size as long as they maintain the defined activity.

[00195] Furthermore, if desired, non-natural amino acids, unnatural amino acids or chemical amino acid analogues can be introduced as a substitution or addition into the polypeptides useful for the invention. Such amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, a-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogues in general.

[00196] The polypeptides useful for the invention can also be differentially modified during or after synthesis, e.g., by biotinylation, benzylation, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. These modifications may serve to increase the stability and/or bioactivity of the polypeptide.

[00197] The polypeptides useful for the invention may or may not comprise a signal peptide. Thus, the polynucleotides encoding these polypeptides may or may not encode a signal peptide. Examples of polypeptides useful for the invention without signal sequences are provided as SEQ ID NO's 17 to 79. Many examples of particular signal peptides which direct the polypeptides to particular cellular locations during expression in a host cell (for example, which facilitate translocation of the polypeptides across a host cell membrane) are known in the art. Particular examples of signal peptides are provided in the specific sequences disclosed herein. The SignalP 4.1 Server (available at http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011) may be used to predict the presence and location of signal peptide cleavage sites in a polypeptide. The polynucleotides and polypeptides may include these specific signal peptides or may not include these specific signal peptides. Thus, the polypeptides may comprise a sequence comprising any one or more of: SEQ ID

NO:1 to SEQ ID NO:4 or SEQ ID NO:9 to SEQ ID NO:12; a polypeptide comprising a sequence which is at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 99.9% identical to any one of SEQ ID NO:1 to SEQ ID NO:4 or SEQ ID NO:9 to SEQ ID NO:12, and a polypeptide comprising a biologically active fragment thereof.

[00198] In one example, the polypeptides may comprise alternative signal peptides in place of the endogenous signal peptides.

[00199] The polypeptides useful for the invention can be produced in a variety of ways, including production and recovery of natural polypeptides, production and recovery of recombinant polypeptides, and chemical synthesis of the polypeptides. In one embodiment, an isolated polypeptide is produced by culturing a cell capable of expressing the polypeptide under conditions effective to produce the polypeptide, and recovering the polypeptide. A preferred cell to culture is a recombinant cell as disclosed herein. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit polypeptide production. An effective medium refers to any medium in which a cell is cultured to produce a polypeptide. Such medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

[00200] The polypeptides of the present invention may be extracted and purified from recombinant cells, such as plant, bacteria or yeast cells, producing said polypeptide by methods known to the person skilled in the art. In one embodiment, the method involves removal of native cell proteins from homogenized cells/tissues/plants etc. by lowering pH and heating, followed by ammonium sulfate fractionation. Briefly, total soluble proteins are extracted by homogenizing cells/tissues/plants. Native proteins are removed by precipitation at pH 4.7 and then at 60°C. The resulting supernatant is then fractionated with ammonium sulfate at 40%

saturation. The resulting protein will be of the order of, at least 50%, or at least 75%, or at least 90%, or at least 95%, pure. Additional purification may be achieved with conventional gel or affinity chromatography.

[00201] In another example, cell lysates are treated with high concentrations of acid e.g. HCl or propionic acid to reduce pH to ~1-2 for 1 hour or more which will solubilise the silk proteins but precipitate other proteins.

[00202] By nature of the inherent coiled coil super secondary or tertiary structure, the polypeptide will spontaneously form the coiled coil secondary structure upon dehydration. As described below, the strength of the coiled coil can be enhanced through enzymatic or chemical cross-linking of lysine residues in close proximity. For example, cross-linking may be promoted according to the method of disclosed in WO2013/120143.

Recombinant Vectors

[00203] A recombinant vector comprising a polynucleotide/nucleic acid encoding a polypeptide defined herein can be used in the production of, for example, a composition of the invention. The polynucleotide/nucleic acid can be inserted into any vector capable of delivering the polynucleotide molecule into a host cell. Such a vector contains heterologous polynucleotide sequences, that is polynucleotide sequences that are not naturally found adjacent to polynucleotide molecules of the present invention and that preferably are derived from a species other than the species from which the polynucleotide molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a transposon (such as described in US 5,792,294), a virus or a plasmid.

[00204] One type of recombinant vector comprises the polynucleotide molecule being operatively linked to an expression vector. The phrase operatively linked refers to insertion of a polynucleotide molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified polynucleotide molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses

or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, endoparasite, arthropod, animal, and plant cells. Particularly preferred expression vectors of the present invention can direct gene expression in bacterial or yeast cells. Vectors can also be used to produce the polypeptide in a cell-free expression system; such systems are well known in the art.

[00205] In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of the polynucleotide. In particular, recombinant molecules include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one recombinant cell such as a recombinant bacterial cell. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, arthropod, plant or mammalian cells, such as, but not limited to, tac, lac, trp, trc, oxy-pro, omp/lpp, rrnB, bacteriophage lambda, bacteriophage T7, T71ac, bacteriophage T3, bacteriophage SP6, bacteriophage SPOl, metallothionein, alpha-mating factor, Pichia alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, Heliothis zea insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells.

[00206] Recombinant molecules of the present invention may also (a) contain secretory signals (i.e., signal segment nucleic acid sequences) to enable an expressed polypeptide of the present invention to be secreted from the cell that produces the polypeptide and/or (b) contain fusion sequences which lead to the

expression of nucleic acid molecules of the present invention as fusion proteins. Examples of suitable signal segments include any signal segment capable of directing the secretion of a polypeptide of the present invention. Preferred signal segments include, but are not limited to, tissue plasminogen activator (t-PA), interferon, interleukin, growth hormone, viral envelope glycoprotein signal segments, Nicotiana nectarin signal peptide (US 5,939,288), tobacco extensin signal, the soy oleosin oil body binding protein signal, *Arabidopsis thaliana* vacuolar basic chitinase signal peptide, as well as native signal sequences of a polypeptide useful for the invention. In addition, the nucleic acid molecule can be joined to a fusion segment that directs the encoded polypeptide to the proteosome, such as an ubiquitin fusion segment. Recombinant molecules may also include intervening and/or untranslated sequences surrounding and/or within the nucleic acid sequences.

Host Cells

[00207] Another embodiment of the present invention includes the use of a recombinant cell comprising a host cell transformed with one or more recombinant molecules, or progeny cells thereof. Transformation of a polynucleotide molecule into a cell can be accomplished by any method by which a polynucleotide molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed polynucleotide molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

[00208] Suitable host cells to transform include any cell that can be transformed with a polynucleotide useful for the invention. Host cells useful for the invention either can be endogenously (i.e., naturally) capable of producing polypeptides defined herein or can be capable of producing such polypeptides after being transformed with at least one polynucleotide molecule encoding the polypeptide. Host cells can be any cell capable of producing at least one protein as defined herein, and include bacterial, fungal (including yeast), parasite, arthropod, animal and plant cells. Examples of host cells include *Salmonella, Escherichia, Bacillus, Listeria, Saccharomyces, Spodoptera,*

Mycobacteria, Trichoplusia, BHK (baby hamster kidney) cells, MDCK cells, CRPK cells, CV-I cells, COS (e.g., COS-7) cells, and Vero cells. Further examples of host cells are *E. coli*, including *E. coli* K-12 derivatives; *Salmonella typhi; Salmonella typhimurium*, including attenuated strains; *Spodoptera frugiperda; Trichoplusia ni* and non-tumorigenic mouse myoblast G8 cells (e.g., ATCC CRL 1246). Additional appropriate mammalian cell hosts include other kidney cell lines, other fibroblast cell lines (e.g., human, murine or chicken embryo fibroblast cell lines), myeloma cell lines, Chinese hamster ovary cells, mouse NIH/3T3 cells, LMTK cells and/or HeLa cells. Other host cells are plant cells such as those available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures).

[00209] Recombinant DNA technologies can be used to improve expression of a transformed polynucleotide molecule by manipulating, for example, the number of copies of the polynucleotide molecule within a host cell, the efficiency with which those polynucleotide molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of polynucleotide molecules of the present invention include, but are not limited to, operatively linking polynucleotide molecules to high-copy number plasmids, integration of the polynucleotide molecule into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of polynucleotide molecules of the present invention to correspond to the codon usage of the host cell, and the deletion of sequences that destabilize transcripts.

[00210] The host cell may be present in a transgenic animal or transgenic plant as described in, for example, WO 2007/038837 and WO 2013/142901.

Chelates

[00211] In one aspect, the polypeptide useful for the invention forms a complex with a chelate comprising a chelating agent and a metal ion.

[00212] As used herein to refer to the association between the chelate and the polypeptide, the term “bound” indicates that the chelating agent forms covalent and/or non-covalent bonds with residues of the polypeptide, forming a complex. As used herein, “bound”, “bind” and the like is understood to encompass covalent and/or noncovalent interactions. For example, the chelating agent may be bound to at least one amino acid of the polypeptide either covalently or non-covalently. The interactions should be specific, that is the polypeptide should bind the chelate through specific means.

[00213] In an embodiment, the chelating agent is not a polypeptide. In a preferred embodiment, the polypeptide and the chelating agent do not form a single polypeptide chain, such as expressed from a single open reading frame.

[00214] The complex may be more or less labile, depending on the specific nature of the chelating agent and the polypeptide in use. In one embodiment, the complex between the chelating agent and the polypeptide is stable enough for the complex to be useful within the needs of the invention.

[00215] As used herein, the term “coordinates” indicates that the metal ion forms a coordinate bond with one or more residues of the polypeptide.

[00216] The term “chelation” refers to the formation of two or more separate coordinate bonds between a polydentate (multiple bonded) ligand and a single central atom, typically a metal ion. The ligands are typically organic compounds, often in anionic form, and can be referred to as chelants, chelators chelating agents, or sequestering agents. Accordingly, the term "chelating agent" as used herein refers to a compound that can form a complex with a metal ion. As used herein, a ligand forming a chelate complex is also referred to as a chelator.

[00217] While chelate complexes typically form from polydentate ligands, as used herein the term chelate also refers to coordination complexes formed from monodentate ligands and a central atom.

[00218] As used herein, the term "chelate" includes a complex of a chelating agent with a mono-, di-, tri-, tetra-, penta-, or hexa-valent cation. The cation may be a metal, for example, but not limited to, a lanthanide or transition metal cation, alkali earth

metal or p-block metal. The chelate may be a simple complex with the cation, involving only non-ionic-bond, non-covalent attractions, or it may be a complex involving ionic bonds and/or other non-covalent attractions. In the latter case, the chelating agent may become ionized by reaction with the cation and/or with a solvent, e.g., water. For example, an oxo-acid-type group of the (neutral) chelating agent, e.g., a phosphonate group or phosphonate ester group, may lose a hydrogen from a hydroxyl thereof, and the resulting oxide moiety might then participate in ionic bonding with the metal ion.

[00219] Many different metal ions are suitable for use as a metal ion in this invention. Preferably, the metal ion is polyvalent and has between 2 to 8 coordination sites, for example, 2, 3, 4, 5, 6, 7 or 8 coordination sites.

[00220] Importantly, the metal ion is selected to allow the compositions described herein to bind a desired target molecule. For example, the metal ion can be any ion that is capable of chelating with heteroatoms such as C, P, N, S, O and the like.

[00221] In some embodiments, the chelated metal ion is an ion of a transition metal.

[00222] In some embodiments, the chelated metal ion is an ion of a p-block metal.

[00223] In some embodiments, the chelated metal ion is selected from the group consisting of an ion of Fe, Sn, Cd, Cr, Mn, Co, Cu, Ru, Zn, Mg, Sc, Rh, Os, Ag, Pd, Zn, Re, Pt, Ti, V, Ni, Mo, Tc, W, and Ir.

[00224] In some embodiments, the metal ion is an alkali earth metal ion. In some embodiments, the alkali earth metal ion is selected from the group consisting of Mg, Be, Ca, Sr, Ba and Ra.

[00225] Exemplary metal ions that can be used in the present invention include zinc (Zn), cadmium (Cd), copper (Cu), nickel (Ni), ruthenium (Ru), platinum (Pt), palladium (Pd), cobalt (Co), magnesium (Mg), barium (Ba), strontium (Sr), iron (Fe), vanadium (V), chromium (Cr), manganese (Mn), rhodium (Rh), silver (Ag), mercury (Hg), molybdenum (Mo) tungsten (W), calcium (Ca), lead (Pb), cerium (Ce), aluminum (Al) and thorium (Th), Tin (Sn), Ruthenium (Ru), Scandium (Sc), Rhodium (Rh),

Osmium (Os), Zinc (Zn), Rhenium (Re), Thallum (Ti), Vanadium (V), Technetium (Tc), and Iridium (Ir).

[00226] The ionic state of the polyvalent metal ions can vary, as is well known. A preferred oxidation or ionic state of a polyvalent metal ion is preferably Zn(II), Cd(II), Cu(I), Cu(II), Ni(II), Ru(II), Ru(III), Pt(II), Pd(II), Co(II), Co(III), Mg(II), Ba(II), Sr(II), Fe(II), Fe(III), Fe(IV), V(III), Cr(II), Cr(III), Mn(II), Rh(III), Ag(I), Hg(II), (Mo(III), Mo(IV), Mo(V), Mo(VI), W(III), W(IV), W(V), W(VI), Ca(II), Pb(II), Ce(III), Al(III), or Th(IV), where the oxidation state is indicated in parenthesis.

[00227] As described above, a chelating agent is a ligand which is capable of forming two or more separate coordinate bonds with a single central atom. The chelating agent is selected to allow the compositions described herein to bind a desired target molecule. For example, the chelating agent may be any chelating agent that is capable of chelating with the desired metal ion and is capable of binding the polypeptide. Importantly, the chelating agent is selected to bind a desired metal ion to allow the compositions described herein to bind a desired target molecule.

[00228] In one embodiment the chelating agent comprises a ring of atoms. Preferably, the chelating agent is a macrocycle. The phrases "macrocycles," "macrocyclic compounds," and "cyclic compounds" are used interchangeably herein to refer to both single cyclic and multi-cyclic compounds having one or more ring structures. The total number of atoms on each of such ring structures may be widely varied, e.g., in a range of from 3 to about 100 or more. Such single cyclic or multi-cyclic compound may further contain one or more linear functional groups, branched functional groups, and/or arched functional groups that bridge across a plane defined by a ring structure. In the case of multi-cyclic compounds having two or more ring structures, any pair of such ring structures may be separated from each another by a non-cyclic spacing structure, or the rings can be in side-by-side relationship to each another, sharing one chemical bond or one atom, or alternatively, the rings may partially overlap with each other, or one ring structure can be enclosed by or intertwined with the other ring. The three-dimensional structures of such compounds can be characterized by any geometric shape, either regular or irregular, including, but not limited to, planar, cylindrical, semispherical, spherical, ovoidal, helical, pyrimidyl, etc.

[00229] In some embodiments, the chelating agent comprises a negatively charged group. In some embodiments, the chelating agent comprises at least one carboxylate group. Without wishing to be bound by theory, it is thought that the carboxylate groups assist binding of the chelating agent to the polypeptide through the formation of non-covalent interactions, for example via electrostatic interactions.

[00230] In some embodiments, the macrocycle comprises at least one pendant group. In some embodiments, the at least one pendant group has a negative charge. In some embodiments, the macrocycle comprises at least one pendant group which is a carboxylate group. Without wishing to be bound by theory, it is thought that the negatively charged pendant group binds to positively charged groups in the polypeptide to assist binding of the chelate (and chelating agent) to the polypeptide.

[00231] Such macrocyclic compounds may include naturally occurring macrocycles and artificial macrocycles. Naturally occurring macrocycles include, but are not limited to, porphryins, including protoporphyrins (e.g. haem b), phytoporphryins (e.g. chlorophyll c) and porphyrinogens (e.g. uroporphryinogen, a biosynthetic precursor), corrins, chlorins, and corphins. Artificial macrocycles include but are not limited to, porphine and phthalocyanines.

[00232] Porphyrins are a group of compounds found in all living matter and contain a tetrapyrrolic macrocycle capable of binding to metals. Haem, chlorophyll and corrins are examples of this class of compounds containing iron, magnesium and cobalt, respectively.

[00233] Suitable metalloporphyrins for use in the present invention that are commercially available through Frontier Scientific, Inc.

[00234] The macrocycles of the present invention include, but are not limited to, porphyrinogens, porphyrins, saphyrins, texaphyrins, bacteriochlorins, chlorins, coproporphyrin I, corrins, corroles, cytoporphyrins, deuteroporphyrins, etioporphyrin I, etioporphyrin III, hematoporphyrins, pheophorbide a, pheophorbide b, phorbines, phthalocyanines, phyllochlorins, phylloporphyrins, phytochlorins, phytoporphyrins, protoporphyrins, pyrrochlorins, pyrroporphyrins, rhodochlorins, rhodoporphyrins, uroporphyrin I, calix[n]pyrroles, calix[n]erines, cycloalkanes, cycloalkenes, cycloalkynes, piperidines, morpholines, pyrrolidines, aziridines, anilines, thiophenes,

quinolines, isoquinolines, naphthalenes, pyrimidines, purines, benzofurans, oxiranes, pyrroles, thiazides, ozazoles, imidazoles, indoles, furans, benzothiophenes, polyazamacrocycles, carbohydrates, acetals, crown ethers, cyclic anhydrides, lactams, lactones, cyclic peptides, phenylthiohydantoins, thiazolinones, succinimides, coronenes, macrolides, carbocyclics, cyclodextrins, squalene oxides, ionophore antibiotics, cyclic bis-N,O-acetals, cyclic disulfides, terpenoids, spirocycles, resorcinarene macrocycles, cyclic oligo(siloxane)s, stannylated cyclic oligo(ethyleneoxide)s, cyclic poly(dibutyltindicarboxylate)s, cyclic poly(pyrrole), cyclic poly(thiophene)s, cyclic poly(amide)s, cyclic poly(ether)s, cyclic poly(carbonate)s, cyclic poly(ethersulfone)s, cyclic poly(etherketone)s, cyclic poly(urethane)s, cyclic poly(imide)s, cyclic poly(decamethylene fumarate)s, cyclic poly(decamethylethylene maleate)s, etc.

[00235] In one embodiment, the chelating agent is selected from the group consisting of porphryins, corrins, chlorins, corphins, porphines and phthalocyanines. In another embodiment the chelating agent is a porphyrin selected from the group consisting of protoporphyrins, phytoporphryins, and porphyrinogens. In another embodiment, the chelating agent is a porphyrin selected from the group consisting of haem *b*, chlorophyll *c* or uroporphryinogen. In another embodiment the chelating agent is corrin, Chlorophyll a, or cofactor F430.

[00236] In one embodiment, the chelating agent is haem. As used herein, the term “haem” refers to a chelate or prosthetic group formed of an iron atom contained in the center of a large heterocyclic organic ring called a porphyrin. Not all porphyrins contain iron, but a substantial fraction of porphyrin-containing metalloproteins have haem as their prosthetic subunit; these are known as hemoproteins or haem proteins. Non-limiting examples of haems are haem A, haem B, haem C, haem O, mesohaems, deuterohaems, synthetic dicyano porphyrins and symmetrical porphyrins (such as, but not limited to, protoporphyrin III).

[00237] Haem proteins are ubiquitous in biological systems carrying out a range of functions such as electron transfer, small molecule transport, catalysis and sensing. Included within the haem protein family are the versatile cytochromes P450, of interest to the pharmaceutical and agrochemical industry and the nitric oxide sensor

protein, soluble guanylate cyclase, which selectively binds nitric oxide over other gases such as oxygen and has been investigated for use in nitric oxide biosensors.

[00238] Haem proteins contain a haem cofactor consisting of a porphyrin ring with an iron coordinated to four nitrogen atoms. The haem cofactor is highly reactive to a wide range of diatomic gases, anions and bases. In biological systems, this reactivity is controlled by the protein environment surrounding the haem cofactor. In most haem proteins, the haem group is held within the protein matrix through hydrogen bonding between the porphyrin ring and amino acid residues such as arginine, tyrosine and serine. In addition, amino acid residues coordinate (forms a chemical bond) with the iron centre. Varying the coordinating ligand is one of the principle ways in which haem proteins regulate the function of the haem cofactor. For example, oxygen transport proteins such as haemoglobin coordinate their metal cofactor using a conserved histidine, the catalytic cytochromes P450 use a conserved cysteine, while electron transport proteins such as cytochrome *c* typically have a bis co-ordinated iron centre with a histidine and methionine residue.

[00239] Generally, the identification of a preferred chelating agent for linking to a metal ion can be made by either first determining the desired chelator to be bonded to the polypeptide backbone, then identifying metal ions that link strongly to that chelator, or the desired metal ion can be first identified with preferred chelator identified subsequently. Thus, preferred chelator are identified and then metal ion candidates are screened for their effectiveness in linking to that chelator. Alternatively, chelators can be screened following identification of a preferred metal ion. Such methods of screening are well known to those skilled in the art.

[00240] In yet a further aspect, the present invention provides a method of producing a composition of the invention, the method comprising (a) combining a candidate polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; a candidate chelating agent and a candidate metal ion (b) determining if the polypeptide, chelating agent and metal ion associate and the chelate is bound to at least one amino acid of the polypeptide. In an embodiment, the method comprises modifying a candidate to polypeptide, such as a silk polypeptide, to introduce at least one, possibly additional, coordinating amino acid. In an embodiment, the method

further comprises testing the ability of the composition to bind and/or modify a target compound.

[00241] The cation, e.g. metal or radionuclide, chosen will depend upon the most appropriate cation, metal ion or isotope for sensing, therapeutic or diagnostic purposes. For example, the introduction of metallo-porphyrins into a polypeptide as described herein. Haem-proteins are capable of performing a large range of functions including oxygen transport, electron transfer/transport and catalysis. Accordingly, metallo-porphyrins can be used for this large range of functions as part of a biosensor according to the present invention. For example, polypeptides comprising iron-porphyrins can be used to bind dioxygen. In one embodiment the metal ion and/or chelator are chosen for their capability of binding a target compound.

Compositions

[00242] In one aspect, the compositions of the present invention comprise a polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; and a chelate comprising a chelating agent and a metal ion; and wherein the chelate is bound to at least one amino acid of the polypeptide.

[00243] In one embodiment, a composition described herein includes more than one polypeptide as described herein. For example, a composition can include two different polypeptides as described herein. In other embodiments, a composition can include a polypeptide as described herein, and a further polypeptide that functions to increase the stability and/or bioactivity of the composition.

[00244] Compositions of the present invention may include an "acceptable carrier".

[00245] Examples of such acceptable carriers include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used.

[00246] The compositions of the present invention may be formed in solution (for instance AmelF3 with a His substitution) or in material form. In an embodiment, the composition is formed in material form.

[00247] As described herein, the silk polypeptides can be fabricated into a range of extremely stable, load bearing materials such as fibres, sponges or films. Accordingly, in some embodiment the polypeptides described herein are provided in a solid material form, and the compositions of the present invention are provided in a solid material form.

[00248] In one embodiment, the polypeptide is formed into a material described herein, and a composition of the invention formed by contacting the material with a chelate. For example, after a polypeptide of the present invention is formed into a material such as a fibre, film, powder, sponge and the like, the material is contacted with a solution containing a chelate under conditions and for a time period sufficient for the chelate to bind to the polypeptide such that the material comprises the composition of the present invention.

[00249] In one embodiment, the polypeptide is formed into a material described herein, and the material is contacted with a chelating agent in the absence of a metal ion under conditions and for a time period sufficient for the chelating agent to bind to the polypeptide and thus form a material comprising a polypeptide-chelating agent complex. The composition of the invention may then be formed by contacting the material comprising polypeptide-chelating agent complex with a metal ion.

[00250] In one embodiment, a composition as described herein is formed into a material such as those described herein. In another embodiment, the composition of the present invention formed by contacting the polypeptide with a chelate under conditions which promote binding of the chelate to the polypeptide. For example, the polypeptide of the present invention is contacted with a solution containing a chelate as disclosed above. The solution contains the chelate at concentrations that favor complex formation, and the polypeptide and chelate are contacted under conditions for a time period sufficient for the chelate to bind to the polypeptide and form a composition of the present invention.

[00251] In another embodiment, the composition of the present invention is formed by contacting the polypeptide with a chelating agent under conditions which promote binding of the chelating agent to the polypeptide such that a polypeptide-chelating agent complex forms. The chelating agent is at concentrations that favor complex

formation, and the polypeptide and chelating agent are contacted under conditions and for a time period sufficient for the chelating agent to bind to the polypeptide and form a polypeptide-chelating agent complex. The composition of the invention may then be formed by contacting the polypeptide-chelating agent complex with a metal ion. The metal ion is at concentrations that favor formation of the composition of the present invention, and the polypeptide-chelating agent complex and metal ion are contacted under conditions and for a time period sufficient to form the composition of the present invention. Optionally, the composition of the present invention may be formed into a material described herein.

[00252] For example, as illustrated in Example 3, a composition of the present invention was formed by combining AmelF3 silk protein and haem *b* in hexafluoroisopranol. The composition was then air dried at room temperature to form a film. The dried film was soaked overnight in 70% methanol for a period sufficient to render the material water insoluble. Without wishing to be bound by theory it is thought that soaking in 70% methanol induces formation of β-sheet structure and helps make the film insoluble in water. Other organic solvents such as ethanol, ethyl acetate and the like can be used to introduce the chelate.

[00253] In some embodiments, the material and/or polypeptide of the present invention is contacted with a solution comprising chelate and a solvent. The amount of chelate can be in excess compared to the amount of polypeptide. In some embodiments, the concentration of the chelate is between about 0.001 to 50 mg/mL. In some embodiments, the concentration of the chelate is between about 0.01 to 10 mg/mL. In some embodiments, the concentration of the chelate is between about 0.1 mg/ml to 5 mg/ml, for example 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml. 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, 3.0 mg/ml, 3.5 mg/ml, 4.0 mg/ml, 4.5 mg/ml or 5.0 mg/ml.

[00254] In some embodiments, the material and/or polypeptide of the present invention is contacted with a solution comprising chelating agent and a solvent. The amount of chelating agent can be in excess compared to the amount of polypeptide. In some embodiments, the concentration of the chelating agent is between about 0.001 to 50 mg/mL. In some embodiments, the concentration of the chelating agent is between about 0.01 to 10 mg/mL. In some embodiments, the concentration of the

chelating agent is between about 0.1 mg/ml to 5 mg/ml, for example 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml. 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, 3.0 mg/ml, 3.5 mg/ml, 4.0 mg/ml, 4.5 mg/ml or 5.0 mg/ml.

[00255] The solvent is one in which the chelate and/or chelating agent is soluble at the desired concentration and which does not adversely affect the material and/or polypeptide. In some embodiments, the solvent may be a polar or non-polar solvent. For example, the solvent may selected from the group consisting of water, alcohol, halogenated alcohols, hydrocarbon, halogenated hydrocarbon, sulfoxide, nitrile, ether, ester, carboxylic acid, ketone and aldehyde. In some embodiments, the solvent is selected from the group consisting of water, pentane, cyclopentane, hexane, cyclohexane, benzene, toluene, diethyl ether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, dimethylformamide, acetonitrile, dimethylsulfoxide, propylene carbonate, formic acid, *n*-butanol, isopropanol, *n*-propanol, ethanol, methanol, acetic acid, chloroform, 2-butanone, hexafluoroisopropanol and mixtures thereof. In some preferred embodiments, the solvent is selected from the group consisting of water, methanol, chloroform, 2-butanone, hexafluoroisopropanol and mixtures thereof. In some [more preferred] embodiments, the solvent is selected from the group consisting of water, methanol, hexafluoroisopropanol and mixtures thereof. For example, the solvent may comprise between 10 - 100% (v/v) methanol in water, such as 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% (v/v). Preferably, the solvent comprises greater than 50% and less than 100% methanol in water (v/v).

[00256] As described above, the polypeptide and chelate are contacted under conditions for a time period sufficient for the chelate to bind to the polypeptide and form a composition of the present invention. The person skilled in the art would understand that the conditions and the time period will vary depending on the concentration of the chelate, the solvent, the desired effect and the like. In some embodiments, the chelate and the polypeptide are contacted for a period of time varying between about 1 second and 7 days. In some embodiments, the chelate and the polypeptide are contacted for a period of time of least 3 seconds, such as but not limited to, 3, 6, 9. 12, 18, 24, 30. 36, 42, 28, 54, 60, 66, or 72 seconds. In some embodiments, the chelate and the polypeptide are contacted for a period of time of

least 3 minutes, such as but not limited to, 3, 6, 9, 12, 18, 24, 30. 36, 42, 28, 54, 60, 66, or 72 minutes. In some embodiments, the chelate and the polypeptide are contacted for a period of time of least 3 hours, such as but not limited to, 3, 6, 9, 12, 18, 24, 30. 36, 42, 28, 54, 60, 66, or 72 hours. In some embodiments, the polypeptide and chelate are contacted at a temperature between about 10°C and 30°C or between about 18°C and 24°C.

[00257] As described above, the polypeptide and chelating agent are contacted under conditions for a time period sufficient for the chelating agent to bind to the polypeptide and form a polypeptide-chelating agent complex. The person skilled in the art would understand that the conditions and the time period will vary depending on the concentration of the chelating agent, the solvent used, the desired effect and the like. In some embodiments, the chelating agent and the polypeptide are contacted for a period of time of least 3 seconds, such as but not limited to, 3, 6, 9, 12, 18, 24, 30, 36, 42, 28, 54, 60, 66, or 72 seconds. In some embodiments, the chelating agent and the polypeptide are contacted for a period of time of least 3 minutes, such as but not limited to, 3, 6, 9, 12, 18, 24, 30, 36, 42, 28, 54, 60, 66, or 72 minutes. In some embodiments, the chelating agent and the polypeptide are contacted for a period of time of least 3 hours, such as but not limited to, 3, 6, 9, 12, 18, 24, 30, 36, 42, 28, 54, 60, 66, or 72 hours. In some embodiments, the polypeptide and chelating agent are contacted at a temperature between about 10°C and 30°C or between about 18°C and 24°C.

[00258] In some embodiments, the composition of the invention may be formed by contacting a polypeptide-chelating agent complex or a material comprising a polypeptide-chelating agent complex with a solution comprising a metal ion and a solvent. In some embodiments, the concentration of the metal ion is between about 0.001 to 50 mg/mL. In some embodiments, the concentration of the metal ion is between about 0.01 to 10 mg/mL. In some embodiments, the concentration of the metal ion is between about 0.1 mg/ml to 5 mg/ml, for example 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml. 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, 3.0 mg/ml, 3.5 mg/ml, 4.0 mg/ml, 4.5 mg/ml or 5.0 mg/ml. The solvent is one in which the metal ion is soluble at the desired concentration and which does not adversely affect the material and/or polypeptide.

For example, the solvent may be a polar solvent or a mixture of polar solvents such as water, alcohols, ammonia and the like.

[00259] As described above, the polypeptide-chelating agent complex and metal ion or a material comprising a polypeptide-chelating agent complex and metal ion are contacted under conditions for a time period sufficient for a composition of the present invention or a material comprising the composition of the present invention to form. The person skilled in the art would understand that the conditions and the time period will vary depending on the surface to volume ratio of the material, the concentration of the metal ion, the solvent used, the desired effect and the like. In some embodiments, the time period varies between about 2 seconds and 7 days. In some embodiments, the time period is at least 1 minute, such as but not limited to, 1, 2, 4, 6, 12, 18, 24, 30, 36, 42, 28, 54, 60, 66, or 72 minutes. In some embodiments, the time period is at least 1 hour, such as but not limited to, 1, 2, 4, 6, 12, 18, 24, 30, 36, 42, 28, 54, 60, 66, or 72 hours. In some embodiments, the temperature is between about 10 °C and 30 °C or between about 18°C and 24°C.

[00260] In one embodiment, composition comprises haem *b* and AmelF3 (SEQ ID NO: 3) without the signal sequence (SEQ ID NO:39). In this embodiment, the chelate is haem *b,* the chelating agent is protoporphyrin IX, the metal ion is iron, and the at least one polypeptide is an AmelF3. The present inventors have demonstrated that when chelator is haem b, the metal ion is iron and the at least one polypeptide is an AmelF3, the tyrosine at position 76 of AmelF3 (SEQ ID NO: 39) forms a co-ordinate bond with iron and haem *b* is bound to at least one positively charged residue of the polypeptide.

[00261] In one embodiment, the composition comprises GA1 and haem *b*. In one embodiment, the composition comprises GA3 and haem *b*.

[00262] In other embodiments, the composition comprises a metal protoporphyrin IX, wherein the metal ion is selected from Fe, Co and Cu, and AmelF3.

[00263] In one embodiment, the composition comprises phthalocyanine tetrasulfonic acid, and AmelF3.

[00264] In other embodiments, the composition comprises a chelate selected from dicyanocobyrinic acid heptamethyl ester or dicyanocobyrinic acid heptamethyl ester, and AmelF3.

[00265] Compositions of the present invention such as those using iron-porphyrin bound to a polypeptide can be used for example, for detecting a target compound, quantifying a target compound, catalysis, electron transfer applications and as antimicrobials.

[00266] The metal or cation and chelator may be combined under any conditions which allow the two to form a complex.

Materials

[00267] In contrast to much work on *de novo* synthesis, the present invention provides protein scaffolds with high stability and which can be easily formed into materials such as fibres, gels, sheets, films, mats, sponges and the like. The present inventors have demonstrated a polypeptide wherein at least a portion of the polypeptide forms a coiled coil structure; and a chelate comprising a chelating agent and a metal ion; and wherein the chelate is bonded to at least one amino acid of the polypeptide is highly stable and able to be formed into materials. Accordingly, the present invention relates to materials comprising compositions of the present invention and methods of producing the materials. Alternatively, the composition of the present invention may be in the form of a material.

[00268] As used herein, the term “stable” refers to the ability of the composition and/or material of the present invention to retain its functional characteristics over time or in different conditions. For example, to retain the ability to bind a detectable compound over a period of time, for example 10 months, 11 months or more.

[00269] As described herein, the compositions of the present invention can be formed as materials that are stable in water. In a preferred embodiment, the materials retain the ability to bind a molecule of interest after a period of time, for example, a week, a month, a year or more. Materials include, but are not limited to, fibres, gels, sheets, films, mats, sponges, powders and the like.

[00270] As used herein, the term "solvent stability" refers the ability of the material to stay insoluble in solvents such as water, SDS (for example 2% SDS), guanadium (for example 8M guanadium) or urea (for example 8M urea). As used herein, "stay insoluble in solvent" means that the material losses than less than 10% of its protein mass after 24 hours at room temperature in the solvent.

[00271] As mentioned herein, in some embodiments of the present invention the polypeptide may be a silk polypeptide. Silk polypeptides are particularly useful for the creation of new materials because of their toughness and strength.

[00272] When the polypeptide is a silk polypeptide, the silk material may be formed from a silk dope. As used herein, the term "silk dope" refers to an aqueous solution comprising silk proteins. Preferably, the silk dope comprises at least 0.05% w/v, more preferably at least 0.1% w/v, and even more preferably at least 0.5% w/v, of a silk protein as defined herein. In an embodiment, silk dope is produced by a method which comprises about 0.5% to about 15% (wt%) silk protein. However, if the further step of increasing the concentration of silk proteins in the silk dope is not performed the more typical yield is about 0.5% to about 4% (wt%) silk protein. Silk dope is amenable to extrusion for the formation of a fibre and/or film casting.

[00273] Methods of making silk dope and materials drawn or extruded from silk dope etc. are disclosed in WO 2011/022771 and WO 2013/120143.

[00274] For example, in one embodiment, the silk dope is produced by a method comprising: i) lysing cells producing one or more silk proteins, ii) solubilising the silk proteins by contacting them with a surfactant or an ionic liquid, and iii) concentrating the silk proteins to produce silk dope, wherein the one or more silk proteins are capable of forming a tertiary structure which comprises a coiled-coil structure. In an embodiment, the surfactant is SDS.

[00275] In one embodiment, a silk dope is provided from which silk materials such as silk fibres, nanofibres, gels, sheets, films, mats, sponges and the like may be produced.

[00276] As used herein, a "silk fibre" refers to filaments comprising silk proteins which can be woven into various items such as textiles. Silk fibres may be formed by

techniques known to the person skilled in the art. Silk fibres useful for the invention have a low processing requirement. The silk proteins useful for the invention require minimal processing e.g. spinning to form a strong fibre as they spontaneously form strong coiled coils which can be reinforced with crosslinks such as lysine crosslinks.

[00277] In some embodiments, silk fibers may spun from solution. Fibrillar aggregates will form from solutions by spontaneous self-assembly of silk proteins useful for the invention when the protein concentration exceeds a critical value. The aggregates may be gathered and mechanically spun into macroscopic fibers according to the method of O'Brien et al. ("Design, Synthesis and Fabrication of Novel Self-Assembling Fibrillar Proteins", in Silk Polymers: Materials Science and Biotechnology, pp. 104-117, Kaplan, Adams, Farmer and Viney, eds., c. 1994 by American Chemical Society, Washington, D.C.).

[00278] In some embodiments, fibers may be spun from solutions having properties characteristic of a liquid crystal phase. The fiber concentration at which phase transition can occur is dependent on the composition of a protein or combination of proteins present in the solution. Phase transition, however, can be detected by monitoring the clarity and birefringence of the solution. Onset of a liquid crystal phase can be detected when the solution acquires a translucent appearance and registers birefringence when viewed through crossed polarizing filters.

[00279] In one fiber-forming technique, fibers can first be extruded from the protein solution through an orifice into methanol, until a length sufficient to be picked up by a mechanical means is produced. Then a fiber can be pulled by such mechanical means through a methanol solution, collected, and dried. Methods for drawing fibers are considered well-known in the art.

[00280] Further examples of methods which may be used for producing silk fibres and/or copolymers are described in US 2004/0170827 and US 2005/0054830.

[00281] Silk fibres may be used in the manufacture of medical devices such as sutures, skin grafts, cellular growth matrices, replacement ligaments, and surgical mesh, and in a wide range of industrial and commercial products, such as, for example, cable, rope, netting, fishing line, clothing fabric, bullet-proof vest lining, container fabric, backpacks, knapsacks, bag or purse straps, adhesive binding

material, non-adhesive binding material, strapping material, tent fabric, tarpaulins, pool covers, vehicle covers, fencing material, sealant, construction material, weatherproofing material, flexible partition material, sports equipment, and, in fact, in nearly any use of fibre or fabric for which high tensile strength and elasticity are desired characteristics.

[00282] As used herein, a "nanofibre" refers to a fibre with a diameter of less than 1000 nm. Nanofibres may be manufactured by techniques known to the person skilled in the art, for example electrospinning (for example, see Wittmer et. al., 2011). In some embodiments, nanofibres cannot be woven into items. Nanofibres may be used in the manufacture of biomaterials that may be used for wound dressings, cell culture and the like.

[00283] As used herein, a "silk film" refers to a film comprising silk proteins. Silk film may be formed by techniques known to the person skilled in the art.

[00284] Silk films may be used in the manufacture of medical devices such as wound dressings, films for biosensor applications such as nitric oxide sensors and in fact, in nearly any use of film for which stability is a desired characteristic.

[00285] As used herein, a "silk sponge" refers to a sponge comprising silk proteins. Silk sponges may be formed by techniques known to the person skilled in the art. Silk sponges may be used for a variety of uses, such as tissue/cell culture scaffolds; catalysis; wound dressings; sensor applications and in fact, in nearly any use of sponge for which stability is a desired characteristic.

[00286] As used herein, a "silk mat" refers to a mat comprising silk proteins. Silk mats may be formed by techniques known to the person skilled in the art. The mat may be an electrospun mat. Silk sponges may be used in a variety of uses, such as tissue/cell culture scaffolds; wound dressings; sensor applications and in fact, in nearly any use of mat for which stability is a desired characteristic.

[00287] In one embodiment, the composition is in the form of a powder.

[00288] In some embodiments, the polypeptide is a recombinant silk protein from aculeate Hymenoptera. Preferably, the recombinant silk protein from aculeate Hymenoptera is a recombinant honeybee silk such as, but not limited to AmelF3.

Recombinant honeybee silk can be manufactured into multiple material forms including fibres and films (Weisman et al., 2010; Sutherland et al., 2011), electrospun mats (Wittmer et al., 2011) and sponges (WO 2011/022771 and WO 2013/120143).

[00289] Preferably, the materials are subject to post-manufacture treatment of some description to render them water insensitive. Examples of post-manufacture treatment include but are not limited to cross-linking, heat treatment or chemical treatment.

[00290] As used herein, a "copolymer" is composition comprising two or more different polypeptides useful for the invention. For example, two or more different silk polypeptides useful for the invention, or two or more silk polypeptides described in WO 2007/038837. As an example, the copolymer (and hence composition) of the invention may comprise AmelF3 and AmelF1, or AmelF3, AmelF1 and AmelF2, or AmelF2, AmelF1 and AmelF4, or GAF3 and AmelF3 etc. Accordingly, the present invention provides a composition comprising a copolymer comprising at least two polypeptides useful for the invention.

[00291] As used herein, "cross-link" is used to refer to both covalent and non-covalent bonds bridging one polymer (such as a polypeptide) chain to another. The person skilled in the art will appreciate that a polymer may fold back on itself and therefore cross-link to itself. Non-covalent crosslinks may include ionic bonds and hydrogen bonds. In polypeptides, cross-links may form between backbone atoms, side chain atoms or both.

[00292] As used herein, "cross-linking" is used to refer to the process of joining one polymer to another or one part of a polymer to another by cross-links.

[00293] In some embodiments, polypeptides, silk fibres, silk films, silk powder, silk sponges and/or copolymers etc of the invention are crosslinked. In one embodiment, the polypeptides, silk fibres, silk films, silk powder, silk sponges and/or copolymers etc are crosslinked to a surface/article/product etc of interest using techniques known in the art. In another embodiment (or in combination with the previous embodiment), at least some silk proteins in the silk fibres, silk films, silk powder, silk sponges and/or copolymers etc are crosslinked to each other. In some embodiments, the silk proteins are crosslinked via lysine residues in the proteins. Such crosslinking can be

performed using chemical and/or enzymatic techniques known in the art. For example, enzymatic cross links can be catalysed by lysyl oxidase, whereas nonenzymatic cross links can be generated from glycated lysine residues (Reiser et al., 1992). In some embodiments, the silk proteins comprise a beta sheet structure in which beta stands are cross-linked to other beta strands in the same or different polypeptide. In these embodiments, the cross-links are non-covalent bonds, preferably hydrogen bonds.

[00294] In some embodiments, the materials are subject to heat-treatment. Heat treatment may comprise heating the material to temperatures that induce formation of lysinoalanine and/or methyllysinoalanine, isopeptide and/or ester cross-links through lysine, serine, threonine asparagine, aspartic acid and/or glutamic acid, residues. Treatment may include heating to around or above 180°C, or heating to lower temperatures (i.e. 120°C) in the presence of a vacuum.

[00295] In one embodiment, the present invention comprises a material formed by a process for heat-treatment of a material comprising the composition of the present invention, the process comprising i) obtaining the material in a solid state, and ii) dry heating the material to a temperature for a sufficient time for the cross-links to form. Preferably, the temperature is at least about 120°C, at least about 120°C or at least about 180°C. In another embodiment, the present invention comprises a material formed by a process for heat-treatment of a material comprising the polypeptide of the present invention, the process comprising i) obtaining the material in a solid state, ii) dry heating the material to a temperature for a sufficient time for the cross-links to form, and iii) contacting the dry heated material with a solution containing a chelate under conditions and for a time period sufficient for the chelate to bind to the polypeptide such that the material comprises the composition of the present invention. Preferably, the temperature is at least about 120°C, at least about 120°C or at least about 180°C. The solution contains the chelate at concentrations that favor complex formation.

[00296] Dry heat treatment of amorphous or helical regenerated silkworm silk materials to above their T_g drives formation of thermally induced β-sheet crystals (Magoshi et al., 1977). Similarly, in regenerated tussah silk heated to 230°C the random coil structure changes to β-sheet, although a-helix content remains fairly

constant (Kweon et al., 2001). Regenerated collagen sponges and fibres are commonly stabilized by a form of heat curing involving heating the material under vacuum to temperatures of 100-120°C for several days (Yannas and Tobolsky, 1967). Heat curing of collagen causes degradation of the collagen, with protein fragmentation increasing with increased temperatures (Gorham et al., 1992). β-sheet structure in coiled coil silk materials has been induced by dry heating to 215°C which is well above the protein's glass transition temperature (Sutherland et al., 2011), however, increased mechanical strength was not observed through lack of cross-links which in hindsight was due to material not having been heated for a sufficient length of time. In contrast, the present inventors have found that exposing material comprising honeybee silk proteins, and/or related coiled coil silk proteins, to high levels of dry heat for a sufficient time promotes the formation of cross-links which confers increased the toughness and/or solvent stability to the heated material. As the skilled person would appreciate, "dry heating" does not necessarily mean that no moisture be present. For instance, dry heating is often performed under normal room humidity conditions such as about 20% to about 80% humidity, or about 30% to about 50% humidity. The processes can rely on heat treatment, the moisture content of the material before heating, and whether the heating step is performed under drying conditions (and the nature of the drying conditions). In light of the teachings herein, a suitable combination of these parameters can readily be determined using standard procedures. If there is any doubt, the benefits can readily be achieved by numerous means such as freeze-drying the material and heating to about 180°C for about 30 minutes, or by heating the material to about 120°C under a vacuum (such as that generated by standard laboratory equipment) for about 48 hours.

[00297] The material which is heated is in a solid state that has too much water will have the effect of boiling the silk proteins. As used herein, "solid state" does not mean that there is absolutely no water in the material at, for example, room temperature or when frozen. In an embodiment, the starting material has a H_20 content of about 1% to about 10%. In an embodiment, the starting material has a H_20 content of less than about 5% or less than about 1%.

[00298] Performing the method under conditions which promote drying counters the above-mentioned boiling effect. Thus, when performed under conditions which promote drying the moisture content of the heated (for example heated to about

100°C to about 120°C) material can be higher than material with a low water content which is heated above 180°C. For example, the closer the vacuum (when used as a drying condition) is to a perfect vacuum the higher the H_20 content can be.

[00299] In one embodiment, the heating is performed in the presence of a vacuum. Broadly, a vacuum is a region with a gaseous pressure much less than atmospheric pressure. The quality of a partial vacuum refers to how closely it approaches a perfect vacuum. Ultra-high vacuum chambers, common in chemistry, physics, and engineering, operate below one trillionth (10) of atmospheric pressure (100 nPa), and can reach around 100 particles/cm.

[00300] In an embodiment, the material is heated in a vacuum to about 100°C. Such a vacuum will probably not be a perfect vacuum. If the vacuum is not particularly strong, and/or a desiccant is not present, it may be necessary to increase the heat, for example to about 120°C to about 150°C. A specific combination of degree of vacuum (pressure) and temperature can readily be determined by the skilled person in view of the present teachings.

[00301] In another embodiment, the heating is performed in the presence of a desiccant (possibly also in the presence of a vacuum). Desiccants are well known to the skilled artisan and are commercially available and include, but are not limited to, silica gel, calcium sulfate, and calcium chloride. If the moisture content of the material which has been obtained is too high (for example the silk proteins are in solution), this can be reduced by drying the material using techniques such as, but not limited to, freeze-drying or precipitation (also known as coagulation).

[00302] Freeze-drying is also referred to in the art as, for example, lyophilization or cryodesiccation. Freeze-drying is achieved by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase. Examples of equipment that can be used to freeze-dry the material include a manifold freeze-dryer, a rotary freeze-dryer and a tray style freeze-dryer. This equipment typically comprises a vacuum pump to reduce the ambient gas pressure in a vessel containing the material and a condenser to remove the moisture by condensation on a surface cooled.

[00303] In an embodiment, the material or solution is frozen at about -20°C, about -30°C, about -40°C, about -50°C or about -60°C or less. In an embodiment, the frozen material or solution is freeze-dried for about 12 to about 48 hours. In a further embodiment, the frozen material or solution is freeze-dried for about 24 hours.

[00304] With regard to precipitation (coagulation), this term refers to converting the starting material (composition comprising silk proteins) from a fluid to a solid state. The material can be precipitated by a variety of techniques such as, but not limited to, the addition of an alcohol or a salt (salting out using, for example, using fluoride, sulfate, hydrogen phosphate, acetate, chloride, nitrate, bromide, chlorate, perchlorate, thiocyanate, ammonium, potassium, sodium, lithium, magnesium, calcium or guanidinium) to a solution comprising the silk proteins, or by reducing the pH of the solution to at least about 5.5, preferably at least about 4.5, or a combination of two or more thereof. In one embodiment, the silk proteins are precipitated in a solution comprising alcohol, the precipitate collected, air dried and used in step ii). Any suitable alcohol can be used, with preferred examples including methanol and ethanol.

[00305] The material can be dry heated using any suitable means known in the art. Examples include, but are not limited to, using an oven, a heat lamp or heat block. As the skilled person would appreciate, dry heat excludes processes which occur in high humidity such as autoclaving.

[00306] Dry heating results in a substantial degree of cross-linking between individual protein, such as silk protein, chains. For silk proteins, the cross-linking appears to be a result of the presence of large number of amino acids in the protein with the potential to form cross-links and the presence of these residues on the surface of the proteins, hence available to form cross-links, when in a coiled coil form. In some embodiments, the cross links are amide cross-links between one or more of glutamine, glutamic acid and aspartic acid residues, and/or ester cross-links between threonine and/or serine with glutamic acid and/or aspartic acid. In an embodiment, there are about two Lys-Glu isopeptide links on average between individual protein molecules.

[00307] The process for heat treatment results in improved properties of the material, for example heat treatment increased toughness and/or solvent stability. The extent of improvement depends on the nature of the material before heating. For example, when compared to an "untreated" sponge the process essentially confers stability when immersed in water. Whilst there is little improvement in water solubility when compared to material previously treated with methanol, heat treatment as described herein essentially confers stability in a solution comprising SDS (for example 2% SDS), urea (for example 8 M urea) or guanadinium (for example 6 M guanadinium), whereas methanol (for example 60% methanol) treated material is soluble in SDS, urea and guanadinium. In yet a further embodiment, heat treatment as described herein increases toughness by at least about 20%, at least about 30%, at least about 40%, at least about 50% when compared to methanol (for example 60% methanol) treated material. In an embodiment, improved toughness and/or solvent stability is assessed when compared to untreated material where the silk proteins have been allowed to associate without additional treatments such as methanol treatment, water annealing or autoclaving.

[00308] As used herein, the term "toughness" refers to the energy required to break the material. Toughness can be measured using any suitable technique known in the art. In one instance, toughness is measured by determining the area under a standard stress- strain curve. In one embodiment, material produced using the method of the invention requires energy to break of at least about 120 MJ/m^3 , or least about 130 MJ/m^3 , or least about 140 MJ/m^3, or least about 150 MJ/m^3, or least about 160 MJ/m^3. In a further embodiment, the method results in at least about a 1.5 fold increase, or at least about a 1.75 fold increase, or at least about a 2 fold increase, in toughness (energy to break) when compared to methanol (for example 60% methanol) treated material.

[00309] In some embodiments, the materials are subject to chemical treatment. In some embodiments, the materials are subject to chemical-treatment with aqueous C_1-C_4 alkanol, for example methanol or ethanol in water. Preferably, the C_1-C_4 alkanol is methanol.

[00310] In one embodiment, the present invention comprises a material formed by a process for chemical-treatment of a material comprising the composition of the

present invention, the process comprising i) obtaining the material in a solid state, and ii) contacting the material with aqueous C_1-C_4 alkanol, such as methanol or ethanol in water, for a sufficient time to render the treated material insoluble. In another embodiment, the present invention comprises a material formed by a process for chemical-treatment of a material comprising the polypeptide as defined herein, the process comprising i) obtaining the material in a solid state, ii) contacting the material with a solution containing a chelate and aqueous C_1-C_4 alkanol under conditions and for a time period sufficient for the chelate to bind to the polypeptide such that the material comprises the composition of the present invention.

[00311] The aqueous C_1-C_4 alkanol contains the chelate at concentrations that favor complex formation, for example where the amount of chelate is in excess. In some embodiments, the aqueous C_1-C_4 alkanol comprises the chelate at a concentration between about 0.001 to 50 mg/mL. In some embodiments, the concentration of the chelate is between about 0.01 to 10 mg/mL. In some embodiments, the concentration of the chelate is between about 0.1 mg/ml to 5 mg/ml, for example 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml. 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml, 3.0 mg/ml, 3.5 mg/ml, 4.0 mg/ml, 4.5 mg/ml or 5.0 mg/ml.

[00312] In some embodiments, the aqueous methanol comprises an amount of C_1-C_4 alkanol which is sufficient to render the treated material less soluble compared to untreated material. In an embodiment, the aqueous C_1-C_4 alkanol comprises at least about 50% methanol, at least about 60% C_1-C_4 alkanol, at least about 70% C_1-C_4 alkanol, at least about 80% C_1-C_4 alkanol or less than about 90% C_1-C_4 alkanol. In an embodiment, the aqueous C_1-C_4 alkanol comprises greater the 50% but less than 100% C_1-C_4 alkanol by volume.

[00313] Without wishing to be bound by theory, it is thought that treating the material and/or composition with aqueous C_1-C_4 alkanol (preferably methanol) generates beta sheet cross-links between the coiled coil polypeptides. It is thought that the formation of the beta-sheet cross-links helps stabilize the material in aqueous solutions. It is thought methanol induces formation of beta-sheets by increasing the hydrophobicity of the solvent and therefore weakening internal protein-protein

hydrophobic interactions. At the same time it though to decrease the availability of water for hydrogen bonding thereby driving protein-protein hydrogen bonding.

[00314] In a preferred embodiment, the aqueous C_1-C_4 alkanol comprises between 50% and 90% methanol in water by volume. It is thought that water acts as a plasticizer, lowering the glass transition temperature of the protein and thus increasing the mobility of the protein and allowing structural rearrangement to occur more readily. Since water is also a solvent for the protein, stabilization of the material requires a compromise to be reached; too much water and the dissolution process dominates, too little or no water and the rate of structural rearrangement becomes unacceptably slow. The present inventors found that materials treated in 50–90% methanol were in an environment with sufficient water to allow protein structural rearrangement but also sufficient methanol to keep the protein precipitated and in the solid form.

[00315] Solubility can be measured using any suitable technique known in the art. In one instance, solubility is measured by assessing the performance of the material in water. Soluble material swells and eventually dissolves after complete immersion in water within 24 hrs.

[00316] As described above, the material was contacted with aqueous C_1-C_4 alkanol for a sufficient time to render the treated material insoluble compared to untreated material. The time depends on the concentration of the C_1-C_4 alkanol in the aqueous solution. In some embodiments, the material is contacted with aqueous C_1-C_4 alkanol for at least about 1 hour, at least about 6 hours, at least about 12 hours, at least about 18 hours, at least about 24 hours, at least about 30 hours, at least about 36 hours, at least about 42 hours, at least about 48 hours, at least about 54 hours or at least about 60 hours. Optionally, the material is dried before use. In an embodiment, the material is dried before use.

[00317] The process for chemical treatment as described herein results in improved properties of the material, at least an increased toughness and/or solvent stability. The extent of improvement depends on the nature of the material before heating. For example, when compared to an "untreated" sponge the process essentially confers stability when immersed in water. While “untreated” sponges

swelled and rapidly dissolved in water, sponges treated with aqueous methanol showed lower levels of swelling and/or dissolution in water.

[00318] Surprisingly, the compositions and/or materials described herein show remarkable stability when stored dry at room temperature. For example, a composition and/or material of the present invention has been demonstrated to be stable when stored dry at room temperature with no deterioration of spectral signal observed over twelve months.

[00319] A solid material form may be used in a number of applications such as a recoverable biocatalytic sponge, a reusable sensing film, or antimicrobial wound dressing and the like.

Binding target compounds

[00320] The present inventors have demonstrated that the compositions, materials and/or copolymers of the present invention are capable of binding target compounds.

[00321] Accordingly, the present invention provides a composition as described herein wherein the composition is capable of binding a target compound. The present invention also provides a composition comprising a binding site for a target compound. The target compound may be in the gas phase or dissolved in a liquid phase.

[00322] The term "target compound" is defined broadly and includes, but is not limited to, small molecules such as oxygen, carbon monoxide, carbon dioxide, nitric oxide and hydrogen cyanide and the like and ions or functional groups such as isocyanide and cyanide hydroxide and the like. For example, target compounds which can be bound by the compositions of the present invention include those selected from the group consisting of oxygen, carbon monoxide, carbon dioxide, compounds having an atom of P, S, or N, and mixtures thereof.

[00323] The present invention is particularly useful for reversibly binding NO and oxygen. The present inventors have demonstrated that the binding of NO with a composition of the present invention results in a detectable change in the composition, and therefore the compositions are suitable for use in biosensor technology.

[00324] In some embodiments, the compositions and/or materials of the present invention are selective for a particular target compound. Selectivity may be altered by altering the chelating agent and/or metal ion present in the composition and/or material and/or the transduction method used (for example, electrochemical versus optical). Selectivity may also be affected by substituting one or more amino acids in the polypeptide sequence.

[00325] The compositions of the present invention can be designed to bind a target compound of interest under the particular conditions of use contemplated. More particularly, the composition will include a chelate which includes a metal ion and a chelating agent, wherein the composition is capable of binding target compound in a target compound-containing environment.

[00326] The term "target compound-containing environment" refers to a medium which includes one or more target compounds, as defined above.

[00327] As discussed above, the chelates may be coordination complexes of any of a variety of transition metals or p-block metals including iron, titanium, tin, manganese, chromium, cobalt, nickel, copper, ruthenium, rhodium, palladium, osmium, iridium, vanadium, zinc, and platinum of a metal ion referred to above. In one embodiment, the metal ion forms complexes and has a first valence state in which the chelate binds a desired target compound. In one embodiment, the metal ion will also have a second valence state in which the chelate is substantially inert to binding the target compound.

[00328] The metal ion and/or the chelating agent in the composition can be varied to perform functions (e.g. target compound binding) not naturally occurring. For example, there are a wide range of artificial porphyrins such as those described herein which are either commercially available or can be readily prepared which can be used to introduce an alternative function. For example cobalt porphyrins are known to have superior nitric oxide affinity while a lower affinity for oxygen, while ruthenium porphyrins can be used as fluorescent oxygen sensors and MRI contrast agents.

Sensors

[00329] The present inventors have demonstrated that the binding of a polypeptide as defined herein to a chelate introduces new properties such as gas binding ability. Accordingly, the compositions of the present invention can be used as biosensors.

[00330] The term "biosensor" when used in the specification is to be understood to mean a system, substrate or device that detects a chemical or biological species with selectivity on the basis of molecular recognition. A biosensor uses a biological recognition element. A chemical or biological species is referred to herein as a target compound. A biosensor uses a composition described herein, as a sensor. A biosensor may use a detectable change in the composition upon binging of a target compound. A biosensor may use specific biochemical reactions to detect molecules by electrical, thermal, optical signals and the like.

[00331] A biosensor typically comprises a biological element of recognition (for example an element capable of binding a target compound). The biosensor may also comprise a signal transducer which measures binding of the target compound to the element of recognition. In some embodiments, the compositions and/or materials of the present invention may be used as an element of recognition in a biosensor.

[00332] For example, haem protein function requires the ability to reversibly reduce and re-oxidise the haem cofactor. Reduction and oxidation can be monitored spectroscopically, since reduction causes a shift in the Soret peak from ~400 nm to higher wavelengths (~420-430 nm). When honeybee silk-haem films are exposed to reducing agents, the Soret peak shifts to 421 nm and there is pronounced splitting in the alpha beta peaks at 527 nm and 558 nm, indicating that Fe^{3+} has been reduced to Fe^{2+}. Similar shifts are observed in the spectrum of haem proteins such as haemoglobin or myoglobin. Reduction is reversed upon the addition of oxidising agents, as demonstrated herein.

[00333] Accordingly, a composition described herein could be used as a nitric oxide biosensor suitable for many applications ranging from monitoring industrial pollutants to biomedical areas (e.g. NO is both a vasodilatory messenger and an endothelial-derived relaxing factor and plays a key role in cellular communication. NO is also produced by inflamed tissue and can be used as a diagnostic tool e.g. NO in breath to diagnose lung inflammation).

[00334] The present inventors investigated the nitric oxide binding of haem *b*-silk material. NO exposure caused a shift in the Soret peak position from 421 nm to 395 nm with increasing NO concentration. The pronounced concentration effect observed demonstrated that a material formed from a composition described herein could be used at a nitric oxide sensor. The films showed remarkable stability, when stored dry at room temperature, no deterioration of spectral signal was observed over at least twelve months.

[00335] The ability to detect a detectable change in the composition allows complicated detection systems (e.g. such as those using marked analytes) or complicated intermediate manipulations to be avoided. The recognition of the target compound by composition may be directly detected by a detectable change. For example, a cascading of events in the transduction of the signal may not be required to detect the detectable change.

[00336] As used herein "detectable change" may be any change in a physical or chemical properties of the composition that serves to indicate to a user the bound or associated state of the target compound and composition, such as, for example, colour, fluorescence, bioluminescence, protein activity, electrochemical (such as conductance or a flow of current) and the like. The indication may, for example, be visually detectable and/or detectable using instrumentation such as a spectrophotometer, for example an absorbance spectrophotometer or fluorescence spectrometer, or a luminometer or a potentiostat for electrochemical measurements. In an embodiment, the detectable change can be detected by electrochemical measurements.

[00337] For example, in some embodiments, the property may be is selected from the group consisting of redox state, electrical conductivity/resistivity, electrochemical, current, potential, capacity, light absorbance, light transmittance, impedance, reflectivity, refractive index, fluorescence, phosphorescence, luminescence, mass as determined by gravimetry or mass-sensitive resonance techniques, heat as determined by calorimetry, conformation and physiological activity of said composition.

[00338] In some embodiments said physical property is light absorbance, light transmittance, reflectivity, refractive index, fluorescence, phosphorescence, or luminescence, and a transducer converts said change in light absorbance, light transmittance, reflectivity, refractive index, fluorescence, phosphorescence, or luminescence into an electrical signal, for example a photometer or spectrophotometer or other device to measure light intensity or any of the aforementioned optical properties.

[00339] In embodiments, wherein said physical property is light absorbance or any of the afore-mentioned other optical properties, the composition may be immobilized on a transparent or reflective electrode, or may be immobilized on an electrically non-conducting transparent or reflective substrate, such as glass.

[00340] In embodiments where the detectable change is electrochemical, the detectable change may be measured using electrochemical methods, such as potentiometric or voltammetric methods.

[00341] In some embodiments, the chelate in the composition or material of the present invention can be a metal-containing group (e.g., a transition metal-containing group, an alkali earth metal or a p-block metal containing group) that is capable of reversibly or semi-reversibly transferring one or more electrons. A number of possible transition metal-containing chelates, an alkali earth metal or p-block metal containing groups can be used.

[00342] The chelate can be capable of undergoing an amperometric or potentiometric change in response to target compound binding.

[00343] In some embodiments, the composition of the present invention may be used as an electron carrier either on its own or in a series of electron carriers. A series of electron carriers may be an electron transport chain, or it may include an electrode. An electron transfer chain transports electrons from a higher to a lower energy level along a series of electron carrier molecules. An electron carrier molecule is a molecule that transfers an electron from a donor molecule to an acceptor molecule. An electron acceptor is a molecule that takes up electrons easily, thereby gaining an electron and becoming reduced, whereas an electron donor is a molecule that easily gives up an electron, becoming oxidised in the process. Therefore a

composition of this invention can be used in a method which involves passing electrons along a sequence of electron carriers, in which each electron carrier is reduced and then oxidised (or vice versa) by electron movement and the sequence of electron carriers includes the protein of this invention. The electron transport chain may comprise natural or synthetic electron carriers. The compositions of this invention can be used in a method involving passing electrons along a sequence of electron carriers, in which each electron carrier is reduced and then oxidised or vice versa by electron movement and the protein of this invention forms part of the sequence of electron carriers. In such a method electrons are generally moved along a gradient of electron carriers with successively lower or higher redox potential.

[00344] An alternative use of the compositions of this invention is as part of an apparatus comprising the composition associated with an electrode in a manner that electrons may be passed from one to the next. The composition may be bound or adsorbed onto the electrode. This includes use of the compositions of this invention in cyclic voltammetry, which is used to provide information about the complex's mid-point potential (redox potential).

Uses

[00345] The present inventors have demonstrated that the compositions of the present invention can bind photosensitising agents such as Zn phthlocyanine tetrasulfonic acid. Accordingly, the compositions and/or materials of the present invention may be used in photodynamic therapy.

[00346] Photodynamic therapy (PDT) is the treatment of malignant tumors with photosensitizers, such as porphyrins and phthalocyanines. Briefly, certain photosensitizers, including porphyrins, metalloporphyrins, and phthalocyanines, localize preferentially in tumor cells. Irradiation of the tissue results in selective cell death of the cells carrying the photosensitizer. Red light in the therapeutically useful range of 600-1200 nm is used. Light in this region of the spectrum has increased transmittance in biological tissue. Both porphyrins and the structurally similar phthalocyanines absorb red light. While porphyrins have been studied more extensively, phthalocyanines have improved absorbance properties and higher extinction coefficients in this region of the spectrum. The photochemistry and

photophysics of porphyrins, metalloporphyrins, and phthalocyanines have been studied in detail. Processes observed include radiationless decay to ground, loss of an axial ligand, energy transfer, electron transfer, formation of singlet oxygen, phosphorescence and fluorescence. The photoprocesses observed in each system depend greatly on the central ligand, normally a metal (2H for porphyrin), the oxidation state of the metal and the axial ligand bound to the metal. A dependence of the photophysical properties on the nature of the macrocycle is also observed. Upon exposure to light the photosensitizing compound may become toxic or may release toxic substances such as singlet oxygen or other oxidizing radicals that are damaging to cellular material or biomolecules, including the membranes of cells and cell structures, and such cellular or membrane damage can eventually kill the cells.

[00347] PDT may also be used to treat or prevent microbial infections. As described above, PDT is based on the use of a photosensitizing molecule that, once activated by light, generates reactive oxygen species ("ROS"). These ROS are toxic to a large variety of prokaryotic and eukaryotic cells (such as the tumour cells described above), including bacteria, mycoplasma, and yeasts. PDT may also comprise the use of trinuclear species [Ru(pc)(pz)2{Ru(bpy)2(NO)}2](PF6)6 (pc = phthalocyanine; pz = pyrazine; bpy = bipyridine) to produce NO and a singlet oxygen as a cancer treatment.

[00348] Accordingly, the invention further comprises methods of killing microbes including Gram-positive and/or Gram-negative bacteria utilizing a composition as describe herein.

[00349] The term microbe is used herein to include microorganisms such as bacteria, fungi, algae, and viruses. An embodiment of this method comprises the steps of providing a composition as describe herein, exposing Gram-positive and/or Gram-negative bacteria to said composition and irradiating the composition for a period of time.

[00350] In one aspect the present invention provides a method of treating a tumour in a subject, said method comprising administering a composition described herein.

[00351] Subjects can be treated by administering to the patient a pharmaceutically effective amount of a composition described herein in the presence of a pharmaceutically acceptable carrier or diluent to produce an effective concentration.

[00352] The compositions according to the present invention are included in the pharmaceutically acceptable carrier or diluent in an amount sufficient to exert a therapeutically useful effect *in vivo* without exhibiting adverse toxic effects on the patient treated.

[00353] There may also be included as part of the composition pharmaceutically compatible binding agents, and/or adjuvant materials. The active materials can also be mixed with other active materials including antibiotics, antifungals, other antivirals and immunostimulants which do not impair the desired action and/or supplement the desired action. The active materials according to the present invention can be administered by any route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

[00354] A preferred mode of administration of the compounds of this invention is oral. Oral compositions will generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the aforesaid compounds may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like. The tablets, pills, capsules, troches and the like may contain the following ingredients: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, corn starch and the like; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; and a sweetening agent such as sucrose or saccharin or flavoring agent such as peppermint, methyl salicylate, or orange flavoring may be added. When the dosage unit form is a capsule, it may contain, in addition to material of the above type, a liquid carrier such as a fatty oil. Other dosage unit forms may contain other various materials which modify the physical form of the dosage unit, for example, as coatings. Thus tablets or pills may be coated with sugar, shellac, or other enteric coating agents. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colourings and flavours.

Materials used in preparing these various compositions should be pharmaceutically pure and non-toxic in the amounts used.

[00355] The solutions or suspensions may also include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methylparabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00356] The concentration of active ingredient in the composition itself will depend on bioavailability of the drug and other factors known to those of skill in the art.

[00357] It is to be noted that dosage values will also vary with the specific severity of the disease condition to be alleviated, and that, for any particular subject, specific dosage regimens should be adjusted to the individual need and the professional judgment of the person administering or supervising the administration of the aforesaid compositions. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

[00358] The compositions of the present invention are prepared as formulations with pharmaceutically acceptable carriers. Preferred are those carriers that will protect the active compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as polyanhydrides, polyglycolic acid, collagen, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

[00359] According to one particular embodiment, the compositions of the present invention can be used to decrease an anti-inflammatory response. More specifically, such anti-inflammatory response may be accompanied by a decrease or reduction in the amount or expression of pro-inflammatory cytokines such as IL-2, IL-17, IL-23, IFN-gamma, IL-6. Such decrease or reduction according to the invention may be a

reduction of about 5% to 99%, specifically, a reduction of about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% as compared to untreated control. In yet another specific embodiment, the composition of the invention may elevate and increase the amount or expression of anti-inflammatory cytokines such as TGF-beta, IL-10, IL-4, IL-5, IL-9 and IL-13. More specifically, the increase, induction or elevation of the anti-inflammatory cytokines may be an increase of about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% as compared to untreated control.

[00360] The compositions of the present invention are useful in a variety of applications including use as a magnetic resonance imaging agent, as a radiation sensitizer, for RNA hydrolysis, and for DNA photocleavage.

[00361] The present inventors have also demonstrated that the compositions of the present invention can function to catalyse the oxidation of substrates. In particular, the present inventors have demonstrated that a composition of the present invention can function as a peroxidase.

[00362] Many enzymes that use an iron (IV) oxoporphyrin radical cation intermediate to catalyze the oxidation of various substrates are known.

[00363] The chelates, in particular the metal ion and/or chelating agent, can be chosen to function as catalysts, for example as peroxidases. For example, the peroxidase activity of a haem b coiled coil silk protein composition of the invention can be used for the bioremediation of phenolic waste.

[00364] In some embodiments, the compositions of the present invention can be used for processes, such as catalytic processes, where cycling of oxidation states is required. For example, the present inventors have demonstrated that the metal ion in a film comprising haem b and AmelF3 can be reversibly oxidised from Fe^{2+} to Fe^{3+}. The chelates, in particular the metal ion and/or chelating agent, can be chosen based on the redox potential of the metal ion.

EXAMPLES

[00365] The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only, and the invention is not limited to these examples, but rather encompasses all variations that are evident as a result of the teachings provided herein.

Materials & Methods

[00366] All standard chemicals used were purchased from Sigma Aldrich. Haem *b* was purchased from Frontier Scientific. UV/Vis absorption measurements were carried out on a SpectraMax M2 plate reader.

Polypeptide synthesis

[00367] Full length, recombinant honeybee silk proteins (AmelF3) without affinity purification tags, were produced by fermentation in *Escherichia coli* as described in Weisman et al. (2010). Full length, recombinant Weaver Ant/Green Ant (*Oecophylla smaragdina*) silk proteins (GA1, GA2, GA3, GA4) without affinity purification tags, were produced by fermentation in *Escherichia coli* following the same procedures as outlined in Weisman et al. (2010).

[00368] Site directed mutagenesis of honeybee silk proteins was carried using methods known to the person skilled in the art. For example, to generate AmelF3 Tyr76Ala, the forward primer 5'-CTCTTGCAGAGGCCGCGTTGCGAGCGTCCG-3' (SEQ ID NO: 80) with the corresponding reverse primer was employed and site directed mutagenesis was carried out using Pfx 50 DNA polymerase from Invitrogen following the Pfx50 manufactures instructions. All AmelF3 mutants were expressed and purified using a similar procedure as the unmodified AmelF3 silk protein. To generate AmelF3 Tyr76His, the forward primer 5'-CTCTTGCAGAGGCCCATTTGCGAGCGTCCG-3' (SEQ ID NO: 81) with the corresponding reverse primer was employed. To generate AmelF3 Ala97His, the forward primer 5'-CCTGAAAAATCATCAACAAGCGCAATTAAACGCCCAGGAAAAGTC-3' (SEQ ID NO: 82) was used.

Example 1: Preparation of recombinant silk-based materials

[00369] Recombinant silk proteins were fabricated into sponges or films using methods described previously (Weisman et al., 2010; Huson et al., 2012; Rapson et

al., 2014). Materials manufactured from silk polypeptides, such as sponges or films, were stored at room temperature until required.

Example 2: Polypeptides having a coiled coil region bind strongly to a chelate comprising a chelating agent and a metal ion

[00370] AmelF3 sponges were prepared as described in example 1. When a solution of haem *b* (0.5mg/mL) in 70% methanol haem *b* is added to honeybee silk sponge (haem *b* in excess), the greyish green haem *b* solution is immediately absorbed into the sponge and within seconds the colour of the sponge changes to red indicating that the haem has become bound to the silk protein (**Figure 1A**). The colour change observed with honeybee silk indicates a change in the coordination of the iron metal centre within the haem group, producing a material with a similar coordination to red haemoglobin. The colour was not removed by extensive washing with 70% methanol, 0.1M HCl or 0.1M NaCl. The retention of the red colour indicates that the haem is strongly bound in the bee silk sponge.

[00371] Figure 1 demonstrates strong binding of a chelate to a polypeptide of the present invention.

Example 3: Polypeptides comprising a coiled coil region form a coordinate bond with the metal ion

[00372] Transparent silk films were prepared as described in example 1 from AmelF3 to monitor the spectral properties of the material using UV/Vis spectroscopy.

[00373] An embodiment of the composition of the present invention was formed by co-drying a solution of AmelF3 and haem *b*. Briefly, 2 mg haem *b* and 20 mg AmelF3 silk sponge was dissolved in 1 mL of HFIP overnight at room temperature. The solution was aliquoted into either a cuvette or 24-well plate and dried at room temperature. The dried film was soaked overnight in 70% methanol to make the film insoluble in water.

[00374] Alternatively, haem *b* was 'leached' into a pre-formed film. The AmelF3 material was formed as described in example 1. The silk material was soaked in a solution of haem *b* (0.5mg/mL; haem *b* in excess) in 70% methanol solution for

between 1 and 48 hours followed by washing with 70% methanol to remove any unbound haem *b* and dried at room temperature.

[00375] Haem proteins have a characteristic Soret peak in their UV/Vis spectrum at ~ 400 nm which is extremely sensitive to changes in the coordination of the iron haem atom. In the case of iron porphyrins such as haem *b*, broad Soret peaks below 400 nm indicate a 4 coordinate iron centre with the iron only coordinated to the porphyrin ring. When the haem group is coordinated to an amino acid (5 coordinate iron centre), the Soret band red shifts to above 400 nm and sharpens.

[00376] UV/Vis absorption measurements were collected between 300 to 600 nm. **Figure 2** shows UV/Vis absorption spectra for a solution of haem *b* (0.05 mg/mL) in water and silk film generated from recombinant honeybee silk protein AmelF3 containing haem b. The UV/Vis spectrum for the film shows a sharp Soret peak at between 400 nm and 410 nm. This is characteristic of Fe coordination to one or two amino acids of the silk polypeptide. In contrast, solutions of haem *b* show a broad peak below 400 nm. This peak is characteristic of uncoordinated Fe in haem b.

[00377] In the case of honeybee silk, the fact that the Fe in the haem *b* coordinates to unmodified silk protein was unexpected and surprising, given that the silk protein does not contain any of the typical coordinating residues such as histidine, cysteine of methionine.

[00378] To determine the nature of the coordinating amino residue the inventors investigated the stoichiometry of haem *b* binding through varying the amount of haem *b* added to the silk protein and using Raman spectroscopy.

[00379] AmelF3 films with different ratios of haem *b* to AmelF3 were prepared by co-drying haem and AmelF3 HFIP solutions. At low haem loadings (haem:protein molar ratios of 1:1 and 1:2), a sharp Soret peak at 404 nm was observed, indicative of all the haem being coordinated (**Figure 3**). As the concentration of haem *b* relative to the protein was increased, the Soret peak broadened and shifted to lower wavelengths indicating an increase in the amount of uncoordinated haem (**Figure 3**). The finding that all haem was coordinated at 1:1 haem:silk ratio suggested that a single amino acid within each silk monomer was responsible for coordination.

[00380] The identity of the coordinating amino acid was investigated using Raman spectroscopy. Raman spectroscopy measures stretching frequencies between the iron centre and the coordinating ligand, these stretching frequencies are indicative of the nature of ligand. Raman spectra were obtained using an inVia confocal microscope system (Renishaw, Gloucestershire, UK) with 754 nm excitation from a Modu-Laser (USA) Stellar-Pro ML/150 Ar ion laser through a x50 (0.75 na) objective. Incident laser power, as measured using an Ophir (Israel) Nova power meter fitted with a PD300-3W head, was 0.59 mW for the silk films and 0.32 mW for the myoglobin powder. Films were held on a mirrored backing while the powder was compressed into a 2 mm cavity cell. A coaxial backscatter geometry was employed. Spectra were collected over the range of 100 to 3200 cm-1 and averaged over at least 20 scans, each with an accumulation time of 20 seconds. The Raman shifts were calibrated using the 520 cm-1 line of a silicon wafer. The spectral resolution was ~ 1 cm-1. All data manipulation was carried out using Grams AI software V 9.1 (Thermo Scientific, USA).

[00381] The Raman spectrum of the silk-haem film excited at 785 nm excitation showed a broad peak centred at 594 cm^{-1} (**Figure 4**), which was specific to the silk-haem film. Haem proteins which have a tyrosine coordinating ligand show similar Fe-Tyr stretches (Nagai et al., 1983 and 1989). Mature recombinant honeybee silk protein 3 contains a single tyrosine residue (Tyr76) located in the core of the predicted coiled coil (SEQ ID NO:39). The Raman spectrum indicated that Tyr76 of AmelF3 was the most likely candidate coordinating to the haem centre.

[00382] To test whether Tyr76 was indeed the coordinating ligand, the present inventors replaced Tyr76 with an alanine using site directed mutagenesis of the AmelF3 silk gene. The UV/Vis spectrum of the Tyr76Ala substituted protein (AmelF3 Tyr76Ala) had a broad Soret peak at 395 nm indicating that the coordination noted in unmodified honeybee silk had been reversed through this single amino acid substitution (**Figure 5A**). When haem *b* was added to sponges prepared using Tyr76Ala silk protein, no pronounced colour change was observed and the green haem *b* colour did not wash out with aqueous methanol indicating that while the haem *b* cofactor bound to the silk protein, the metal ion was not coordinated to the polypeptide.

[00383] This data indicates that recombinant honeybee silk can be used as a stable protein scaffold for haem protein engineering. A tyrosine residue in the core of the coiled-coil was determined to be coordinating to the iron haem centre. Site directed mutagenesis has demonstrated that it is possible to control the coordination of the haem centre.

Example 4: The polypeptide can be modified using site-directed mutagenesis to control co-ordination of the metal ion

[00384] The present inventors also mutated Tyr76 to a histidine and investigated the effect the single amino acid substitution had on binding to haem *b*. As indicated by the UV/Vis spectrum AmelF3, Tyr76His bound haem *b* via a co-ordinate bond (**Figure 5B**) in solution and when formed into a film. The sharp peak observed at around 400 nm is indicative of the coordination of an amino acid residue to the iron haem centre. This data indicates that polypeptides having a coiled coil region can be used as a stable protein scaffold for binding to a chelating agent. Site directed mutagenesis has demonstrated that it is possible to alter binding to the chelating agent.

Example 5: Binding of a chelate comprising a chelating agent and a metal ion to a polypeptide having a coiled coil region is not unique to beesilk polypeptide

[00385] Transparent films from the Green Ant (*Oecophylla smaragdina*) (GA1-4) silk proteins were prepared as described above. Haem *b* in 70% methanol was leached into the preformed Green Ant silk film as described at Example 3. UV/Vis spectra were collected for Green Ant silk film after haem *b* had been leached. For GA1 and GA3, a strong signal was observed at around 400 nm demonstrating that these proteins strongly bound haem (**Figure 6**). This data show that other polypeptides having a coiled coil structure are capable of binding a chelate comprising a chelating agent and a metal ion.

[00386] Transparent film from praying mantis silk were prepared as described above. Haem b was leached into the preformed films as described in Example 3. UV/Vis spectra were collected. A strong signal was observed at ~400 nm demonstrating that these proteins strongly bound haem (**Figure 7**). This data show

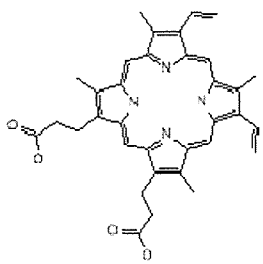
that other polypeptides having a coiled coil structure are capable of binding a chelate comprising a chelating agent and a metal ion.

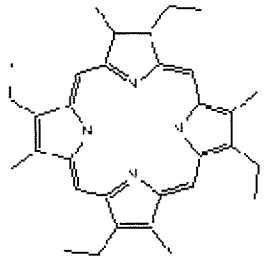
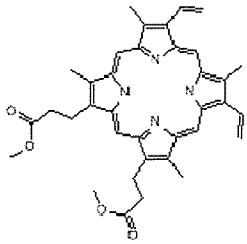
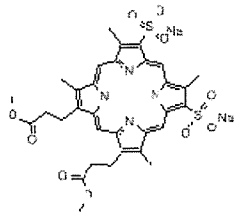
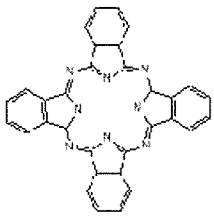
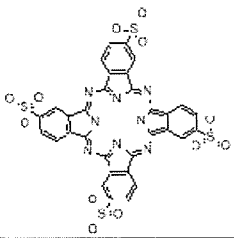
Example 6: Coiled coil silk polypeptides bind other macrocycles in addition to haem *b*

[00387] The ability of sponges generated from recombinant AmelF3 to bind a range of macrocyles was tested by adding a solution of the macrocmacrocycle containing an excess of the macrocycle to be tested to the AmelF3 sponge and observing the colour of the sponge and whether the sponge retained colour after washing. The strength of macrocycle binding was assessed qualitatively by assessing the intensity of the colour retained by the sponge after washing. Strong binding to the sponge refers to the finding that the macrocycle cannot be washed out with aqueous methanol, organic solvents, 0.1 M HCl or 0.1 M NaOH. Some of the macrocycles were noted to weakly bind the macrocycle – in this case a faint colouration of the sponge was observed and UV-Vis spectra showed weak absorption peaks.

[00388] The sponges were able to bind a number of porphyrins and phthalocyanines as summarised in Table 2 below.

Table 2. Binding properties of different macrocycles to materials generated from recombinant honeybee silk proteins.

Macrocycle	Binding properties
Porphyrins	
Protoporphyrin IX (PPIX) 	Strongly binds to silk

Etioporphyrin I 	Does not bind
Protoporphryin IX dimethyl ester 	Does not bind
Deuteroporphyrin IX 2,4-disulfonic acid dimethyl ester 	Weak binding
Metal protoporphyrin IX containing either Fe, Co and Cu	Strongly binds
Phthalocyanines	
Phthalocyanine 	Does not bind
Phthalocyanine tetrasulfonic acid 	Strongly binds

[00389] These results indicate that, in the absence of a metal within the macrocycle, a charge in the macrocycle (such as a carboxyl group e.g. PPIX or sulfonate group e.g. phthalocyanine) is preferred to allow the macrocycle to bind to a residues of the opposite charge in the silk protein. If no charge is present e.g. Phthalocyanine or etioporphryin I, no binding is obtained.

[00390] This data also shows that the metal ion is not required for strong binding between the polypeptide and the chelate. Therefore, interactions between the polypeptide and the chelating agent are sufficient for strong binding.

[00391] These results suggest that an appropriately located negative charged residue in the silk will bind a macrocycle with a positive charge, and that an appropriately located positive charged residue in the silk will bind a macrocycle with a negative charge.

Example 7: Coordination of a metal ion contained within a chelating agent with at least one amino acid of the polypeptide is sufficient for binding

[00392] Films generated from recombinant honeybee silk protein AmelF3 containing dicyanocobyrinic acid heptamethyl ester were prepared as described in example 3. UV/Vis spectrum were recorded for a film generated from recombinant AmelF3 containing dicyanocobyrinic acid heptamethyl ester and a solution of dicyanocobyrinic acid heptamethyl ester without AmelF3 (**Figure 8**).

[00393] Dicyanocobyrinic acid heptamethyl ester was found to strongly blind to AmelF3 in film. The shift in the position of the peaks from 370-360 and changes in the 500-600 nm when dicyanocobyrinic acid heptmethyl ester is bound to the silk protein suggests that the Tyrosine residue is coordinating to the cobalt centre. When a solution of dicyanocobrynic acid heptamethyl ester was added to sponges prepared from AmelF3 Try76Ala and washed extensively with 70% methanol the pink colour washed out of the sponge indicating that AmelF3 Try76Ala did not bind to dicyanocobyrinic acid heptamethyl ester.

[00394] This indicates that binding of dicyanocobyrinic acid heptamethyl ester takes place through coordination of the tyrosine residue to the cobalt metal centre

alone. There is unlikely to be binding between the chelating agent and the polypeptide. Therefore, a coordinate bond between the polypeptide and the metal ion is sufficient for binding. Dicyanocobyrinic acid heptmethyl ester has been introduced to both preformed films and also sponges (data not shown).

Example 8: A composition according to the invention can be reversibly reduced and oxidised

[00395] Haem protein function such as gas binding requires reduction of the haem group from Fe^{3+} to Fe^{2+}, while catalysis requires cycles of reduction and re-oxidisation. Reduction and oxidation of Fe can be monitored spectroscopically, since reduction causes a shift in the Soret peak from ~ 400 nm to higher wavelengths (~ 420-430 nm, depending on the coordination system).

[00396] Freshly prepared sodium dithionite (100 mM) in 50 mM phosphate buffer (pH 7) was used as the reducing agent. 10 – 100 µL was added to a 50 mM phosphate buffer (pH 7) covering the porphyrin-AmelF3 film. Sodium persulfate was used at the oxidising agent in a similar manner to sodium dithionite.

[00397] When the AmelF3-haem films were exposed to reducing agents, the Soret peak shifted to 421 nm indicating that Fe^{3+} has been reduced to Fe^{2+} (**Figure 9**). Reduction was reversed upon the addition of an oxidising agent (**Figure 9**). This data demonstrates that haem-silk materials can be used for catalytic processes, for example catalytic processes where cycling of oxidation states is required.

Example 9: A composition according to the invention can bind nitric oxide

[00398] Artificial haem materials hold potential for sensing a range of gases and volatile organic compounds. For example, artificial haem proteins are excellent candidates for sensing nitric oxide (NO), for which there is a need in many fields, ranging from monitoring industrial pollutants to clinical diagnosis and biomedical research. The inventors therefore investigated the nitric oxide (NO) binding ability of the silk-haem b material.

[00399] AmelF3-haem b film was used as the porphyrin-silk film. Freshly prepared sodium dithionite (100 mM) in 50 mM phosphate buffer (pH 7) were used as the reducing agent. 10 – 100 µL was added to a 50 mM phosphate buffer (pH 7) covering the porphyrin-silk film. Sodium persulfate was used at the oxidising agent a similar

manner to sodium dithionite. Diethylamine 2-nitrosohydrazine sodium hydrate (NONOate) was dissolved in 50 mM phosphate (pH 7) to generate 1.5 mol equivalent of dissolved NO. Aliquots of the diethylamine NONOate solution was added to a dithionite reduced film. Changes in the UV/Vis spectrum were monitored to determine redox states and measure NO binding (**Figure 10A and 10B**). NO exposure caused a shift in the Soret peak position from 421 nm to 395 nm with increasing NO concentration.

[00400] Exposure to increasing NO concentration caused the Soret peak to shift from 421 nm to 395 nm. From 0 – 7 μM, there was a linear relationship between the decrease in absorbance at 421 nm and the concentration of NO, with a limit of detection for NO of 1 μM. NO binding was found to be reversible and the silk-haem *b* films.

[00401] This data shows compositions of the present invention are capable of binding a target molecule (e.g. NO), and that target molecule binding results in a detectable change in the composition.

Example 10: A composition according to the invention can be used as a catalyst

[00402] The present inventors investigated whether haem silk materials generated from recombinant honeybee silk containing bound haem *b* can act as peroxidases. This activity was demonstrated using a modification of the Worthington assay (www.worthington-biochem.com/hpo/assay.html). The assay uses 4-aminoantipyrine as hydrogen donor. The reaction rate is determined by measuring an increase in absorbance at 510 nm resulting from the oxidative coupling of aminoantipyrine with phenol with decomposition of hydrogen peroxide (**Figure 11**).

Example 11: A composition according to the invention can be used to bind ZnPc which has antimicrobial activity

[00403] Photodynamic therapy uses nontoxic, light-sensitive compounds that, when exposed to selective wavelengths of light become toxic to microbes (bacteria, fungi and viruses), targeted malignant cells or other diseased cells. Photodynamic therapy

requires a photosensitizer, light source and tissue oxygen. The light excites the photosensitizer which leads to the production of toxic reactive oxygen species.

[00404] Macrocycles that can act as photosensitizer can be bound within protein films generated from recombinant honeybee silk (**Figure 12**). It can be expected that these materials ideally suited for photodynamic therapy.

Example 12: The composition of the present invention may comprise different metal ions

[00405] **Figure 13** shows the metal ion of the chelate may be varied. Chelates including copper (CuPPIX), cobalt (CoPPIX) and haem *b* (FePPIX) were introduced into AmeIf3 silk films. The ability to vary the metal ion can be used to alter the function of the composition, for example, different target compound binding.

Example 13: A composition according to the invention is stable for at least a year

[00406] AmeIF3-haem *b* films showed remarkable stability; they could be stored dry at room temperature for at least one year, with no deterioration in NO binding ability (**Figure 14**). This data also demonstrates the materials showed no deterioration of spectral signal over ten months.

Example 14: A composition according to the invention is stable in organic solvents

[00407] The present inventors have demonstrated that the silk films and sponges are stable in a variety of different organic solvents. UV/Vis spectra of AmeIF3 silk film in different solvents were recorded. **Figure 15** shows silk materials are stable in a variety of aqueous and non-aqueous solvents such as water, chloroform, ethyl acetate and ethanol. This data demonstrates compositions of the present invention can be used in catalytic applications where non-aqueous solvents are required.

Example 15: A composition of haem-silk materials is compatible with electrochemical signal transduction

[00408] A glassy carbon electrode was modified with carbon nanotubes by drying a dispersion of carbon nanotubes (single walled; 1-5 μm) in DMF. A film of AmeIF3 was cast on top of the nanotube layer by drying a solution of AmeIF3 in water (10mg/ml).

Haem b was leached into the AmelF3 as described previously from a 70% methanol solution. A reversible voltammetric response was noted for the haem b –AmelF3 samples which are not observed in the absence of heme (dotted line) or without AmelF3 silk (dashed-dotted line) and can be attributed to the Fe3+/Fe2+ redox couples. **Figure 16)**. This difference demonstrates that silk-haem materials are compatible with electrochemical signal transduction methods.

Example 16: A composition of haem-silk materials can be used to detect nitric oxide

[00409] Using the electrode prepared as described in Example 15, upon the addition of nitric oxide a pronounced catalytic current is noted (**Figure 17**). This catalytic current is attributed to the oxidation of nitric oxide by haem-silk materials can be used to determine the nitric oxide concentration in samples.

Example 17: A composition of haem-silk materials can be used to detect oxygen

[00410] A haem-silk electrode was prepared as in Example 15. The electrode was held at a constant potential (-300 mV vs Ag/AgCl) under anaerobic conditions (through purging with argon gas). Aliquots of aerated buffer solution were added to introduce oxygen at varying concentrations. The sharp increase in catalytic current is due to the reduction of oxygen by haem-silk materials demonstrating that these materials can be used as an oxygen sensor (**Figure 18**).

Example 18: An additional haem binding site can be introduced into AmelF3

[00411] A coordinating His residue was introduced into AmelF3 with Y76A in the a-position of the coiled-coil. The resulting protein (Y76A A97H) showed a sharp Soret peak at 413 nm indicative of histidine coordination (**Figure 19**). This demonstrates that additional binding sites can be introduced in coiled-coil polypeptides.

Example 19: Demonstration of increased heme binding in AmelF3

[00412] A coordinating His residue (His97) was introduced into AmelF3 with Y76H substitution in the a-position of the coiled-coil. Y76H shows a distinct 1:1 ratio of heme binding to silk protein, indicated by the broadening of the Soret peaks a heme ratios above 1:1 and a shift in the position of the peak maxima (**Figure 20**). No change in both the shape and the position of the Soret peak was noted for Y76H

A97H indicating that an extra heme binding site had been introduced and the modified AmelF3 was now able to bind two heme cofactors.

[00413] It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

[00414] The present application claims priority from AU 2014904612 filed 17 November 2015, the entire contents of which are incorporated herein by reference.

[00415] All publications discussed and/or referenced herein are incorporated herein in their entirety.

[00416] Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. A composition comprising:
   a polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; and
   a chelate comprising a chelating agent and a metal ion; and
   wherein the chelate is bound to at least one amino acid of the polypeptide.

2. A composition of claim 1, wherein the chelating agent is bound to at least one amino acid of the polypeptide.

3. A composition of claim 1 or claim 2, wherein the metal ion is bonded to at least one amino acid of the polypeptide by a co-ordinate bond.

4. A composition of any one of claims 1 to 3, wherein the portion of the polypeptide that has a coiled coil structure comprises at least 35 amino acids.

5. A composition of claim 4, wherein the portion of the polypeptide that has a coiled coil structure comprises at least 63 amino acids.

6. A composition of any one of claims 1 to 5, wherein at least 20% of the amino acids in the coiled coil structure are alanine residues.

7. A composition of any one of claims 1 to 6, wherein the polypeptide comprises about 9 to about 30 heptad repeats.

8. A composition of any one of claims 3 to 7, wherein the at least one amino acid bound to the metal ion by a co-ordinate bond is a Tyr, Cys, His, Met, Lys, Glu or a non-natural amino acid.

9. A composition of any one of claims 1 to 8, wherein at least one amino acid residue bound to the chelating agent is a charged amino acid residue.

10. A composition of any one of claims 1 to 9, wherein the chelating agent comprises a ring of atoms.

11. A composition of any one of claims 1 to 10, wherein the chelating agent is selected from the group consisting of porphryins, corrins, chlorins, corphins, porphines and phthalocyanines.

12. A composition of any one of claims 1 to 11, wherein the metal ion is an ion of a transition metal, alkali earth metal or p-block metal.

13. A composition of any one of claims 1 to 12, wherein the metal ion is selected from the group consisting of an ion of Fe, Sn, Cd, Cr, Mn, Co, Cu, Ru, Zn, Mg, Sc, Ru, Rh, Os, Ag, Pd, Zn, Re, Pt, Ti, V, Ni, Mo, Tc, W and Ir.

14. A composition of any one of claims 1 to 13, wherein the composition is capable of binding a target compound.

15. A composition of any one of claims 1 to 14, wherein the composition comprises a binding site for a target compound.

16. A composition of claim 14 or claim 15, wherein the target compound is selected from the group consisting of oxygen, carbon monoxide, carbon dioxide, hydrogen peroxide, compounds having an atom of P, S, or N, and mixtures thereof.

17. A composition of any one of claims 14 to 16, wherein the target compound is NO.

18. A composition of any one of claims 1 to 17, wherein the composition comprises more than one polypeptide.

19. A material comprising a composition according to any one of claims 1 to 18, wherein the polypeptides are crosslinked by ionic bonds, Hydrogen-bonds, covalent bonds or a combination thereof and the material is insoluble in water.

20. A material of claim 19, wherein the material is a silk fibre, film, powder or sponge.

21. A copolymer comprising a composition according to any one of claims 1 to 18 and a further polypeptide, wherein at least a portion of the further polypeptide has a coiled coil structure.

22. A copolymer of claim 21, wherein at least some of the polypeptides are crosslinked.

23. A copolymer of claim 22, wherein at least some of the residues of the polypeptides are covalently crosslinked.

24. A sensor for detecting a target compound comprising a composition of any one of claims 1 to 18, and/or a material of claim 19 or claim 20, or copolymer of any one of claims 21 to 23.

25. A sensor according to claim 24, wherein the composition comprises a binding site for the target compound, and wherein binding of the target compound results in a detectable change.

26. A sensor according to claim 25, wherein the detectable change is a colour, spectrophotometric, fluorescent or electrochemical change.

27. A sensor according to claim 26, wherein the spectrophotometric change is a change in the Soret peak, or a change in at least one spectrophotometric peak with a wavelength between 500 and 600 nm.

28. A method of binding a target compound, said method comprising the steps of (a) providing a composition of any one of claims 1 to 18 and/or a material according to claim 19 or claim 20, and (b) contacting the composition with a target compound under conditions for binding said compound to said composition.

29. The method of claim 28 which further comprises detecting binding of the target compound by detecting a change in the composition and/or target compound upon binding.

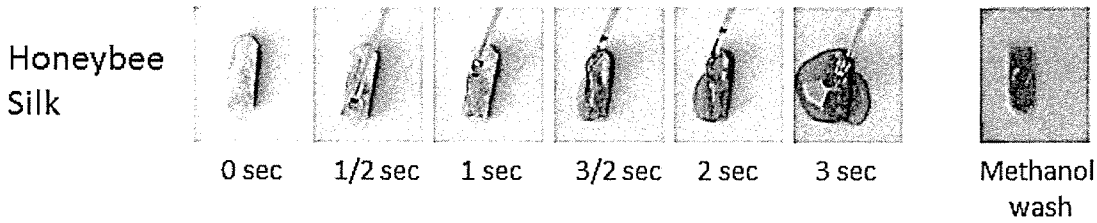
30. A method for producing a biosensor, the method comprising providing a polypeptide wherein at least a portion of the polypeptide has a coiled coil structure; and contacting the polypeptide to a chelate comprising a chelating agent and a metal ion under conditions for binding said chelate to at least one amino acid of said polypeptide.

31. A method of claim 30, wherein the metal ion is bound to the at least one amino acid of the polypeptide by a co-ordinate bond.

32. A method of claim 30, wherein the chelating agent is bound to the at least one amino acid of the polypeptide.

Figure 1

A



B

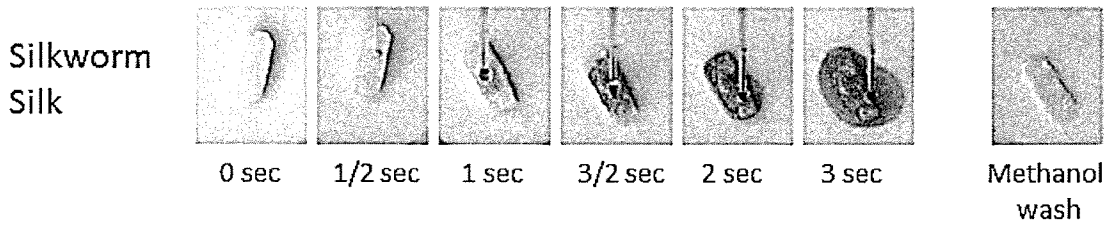


Figure 2

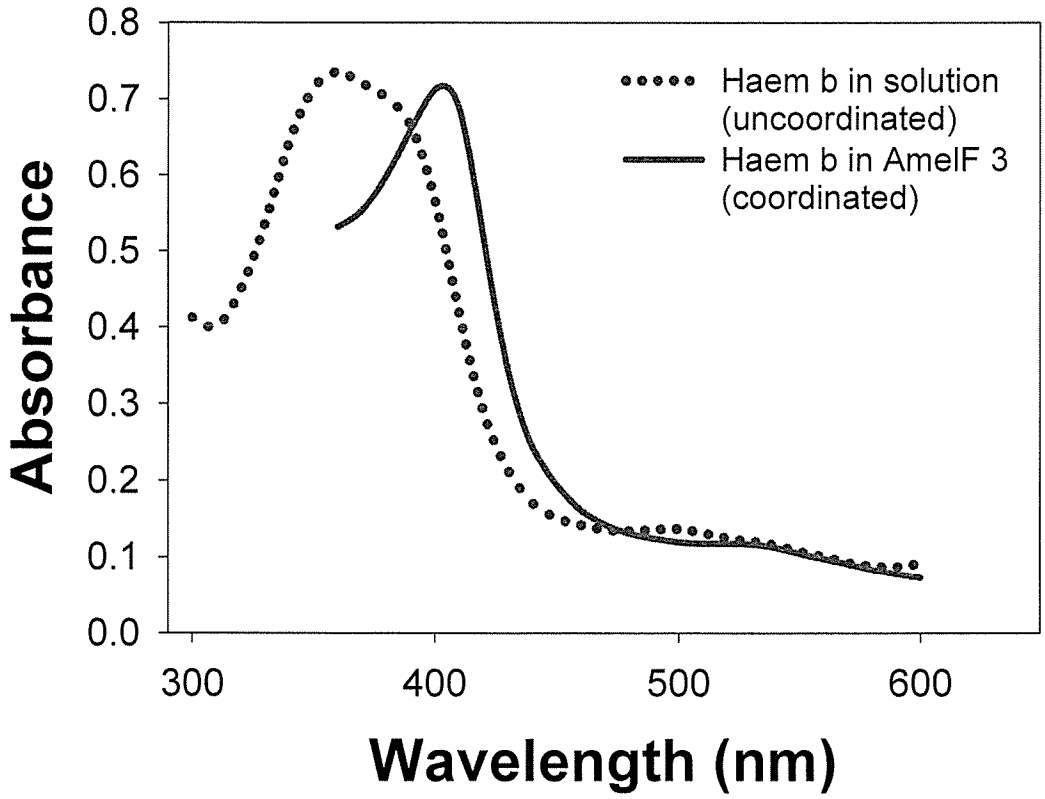


Figure 3

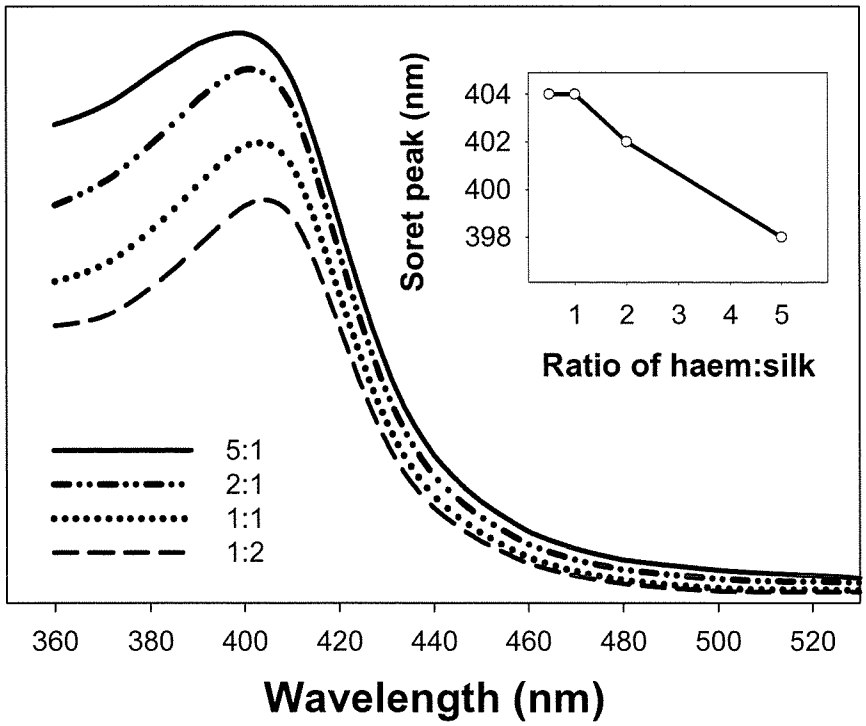


Figure 4

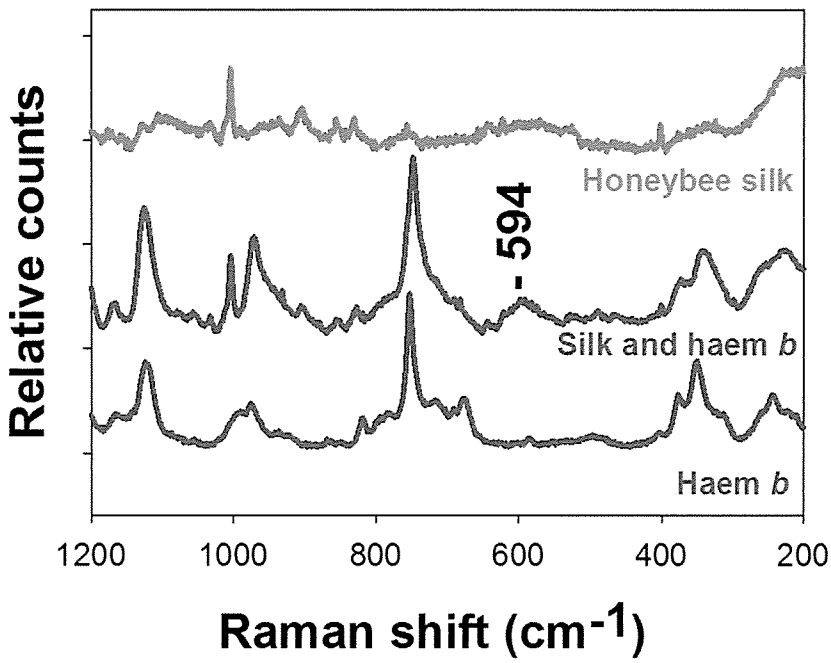
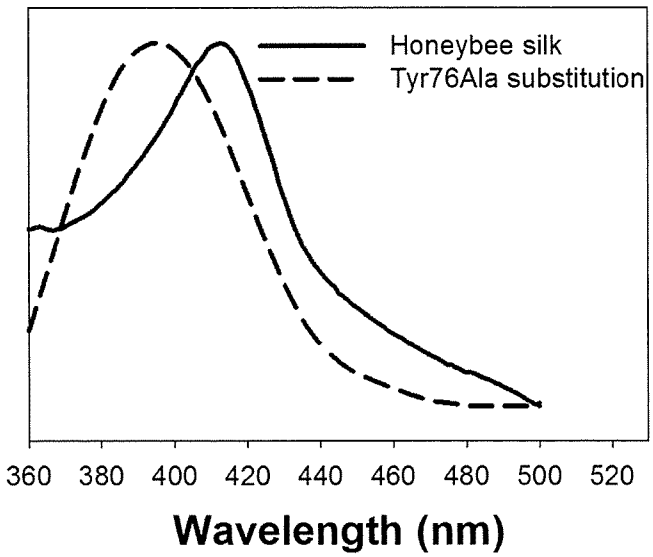


Figure 5

A


Honeybee silk
Tyr76Ala substitution
360 380 400 420 440 460 480 500 520
Wavelength (nm)

B

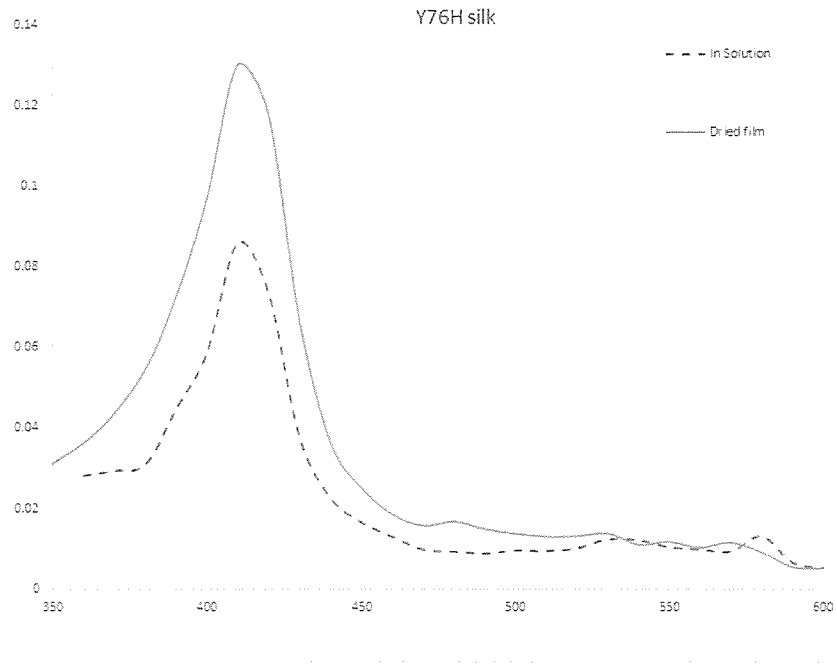

Y76H silk
In Solution
Dried film
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
350
400
450
500
550
600

Figure 6

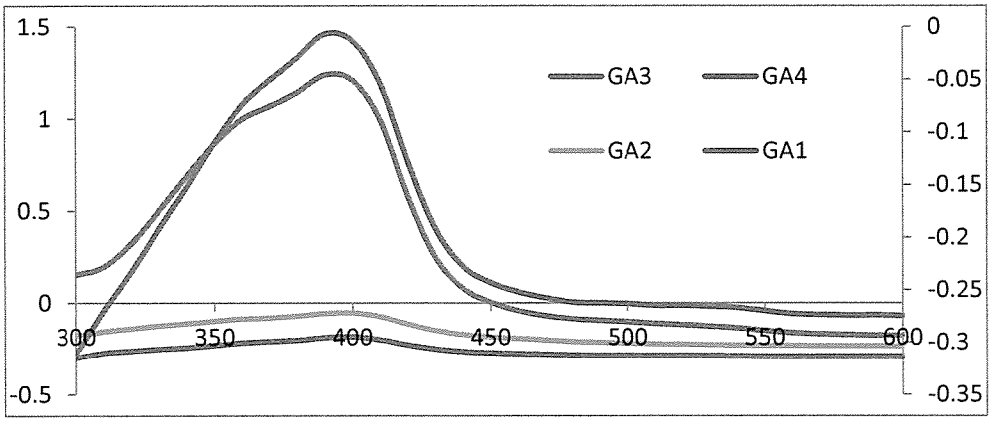


Figure 7

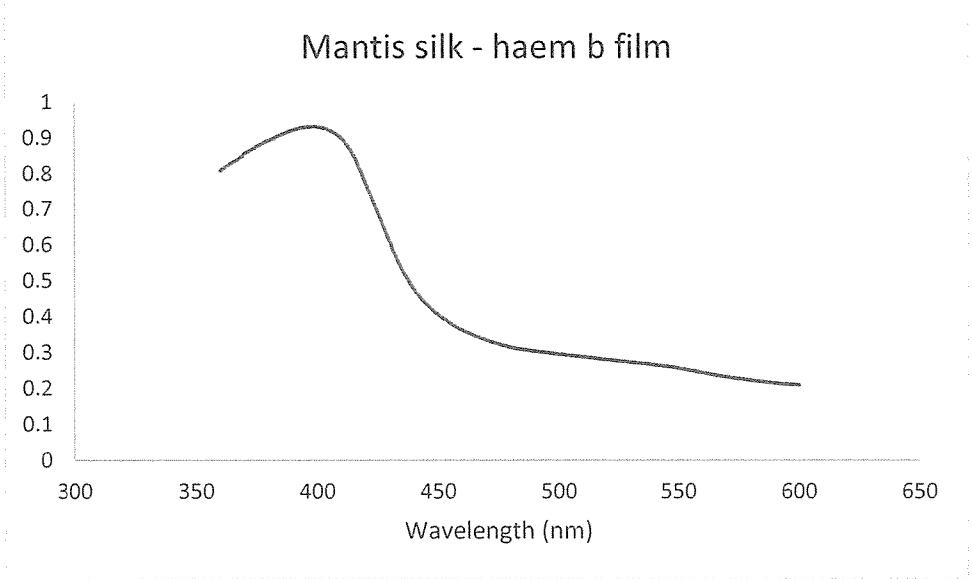


Figure 8

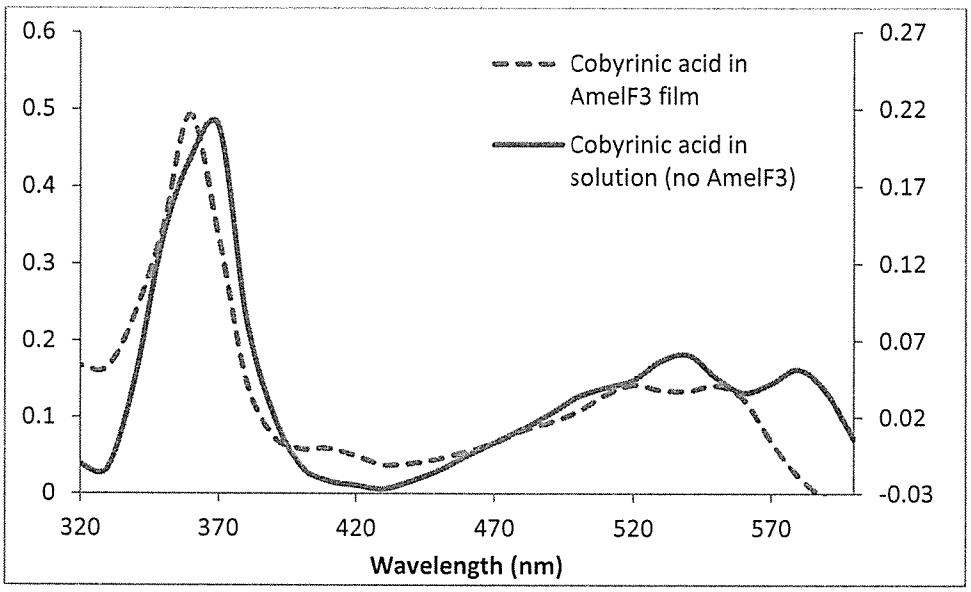

0.6
0.5
0.4
0.3
0.2
0.1
0
0.27
0.22
0.17
0.12
0.07
0.02
-0.03
320
370
420
470
520
570
Wavelength (nm)
Cobyrinic acid in AmelF3 film
Cobyrinic acid in solution (no AmelF3)

Figure 9

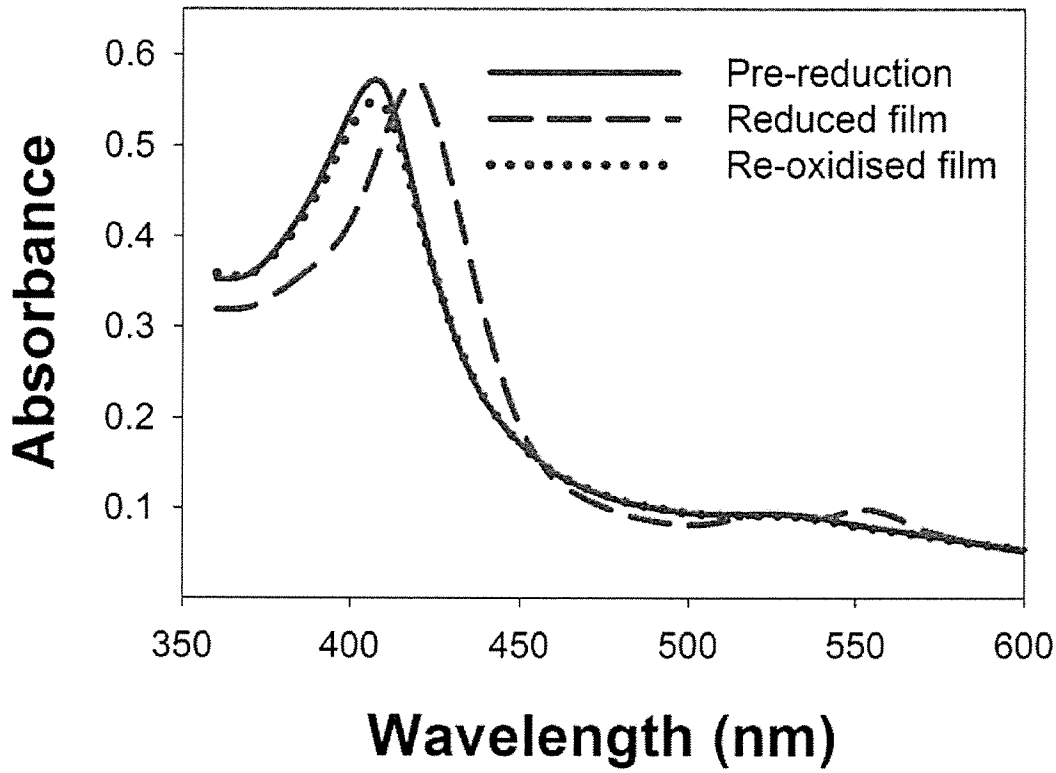
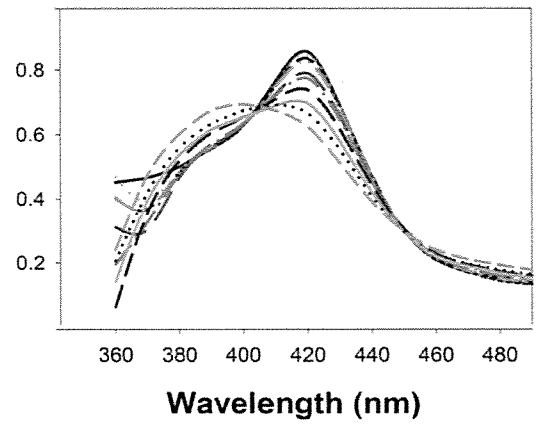

Absorbance
0.6
0.5
0.4
0.3
0.2
0.1
350
400
450
500
550
600
Wavelength (nm)
Pre-reduction
Reduced film
Re-oxidised film

Figure 10

A


0.8
0.6
0.4
0.2
360
380
400
420
440
460
480
Wavelength (nm)

B

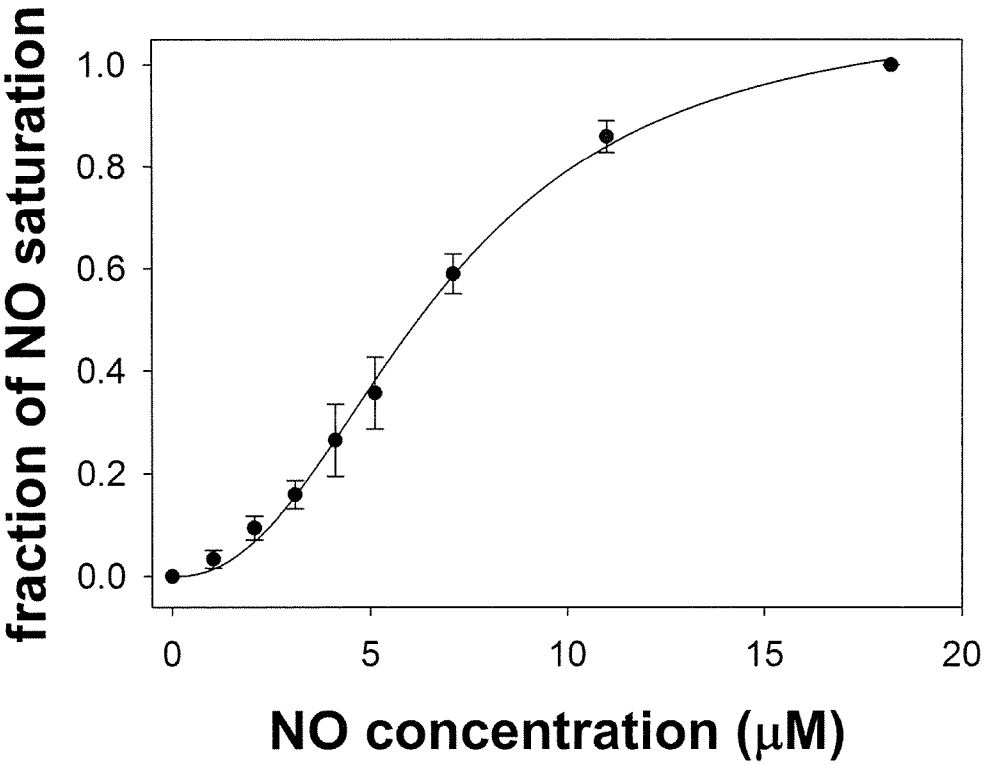

fraction of NO saturation
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
NO concentration (μM)

Figure 11

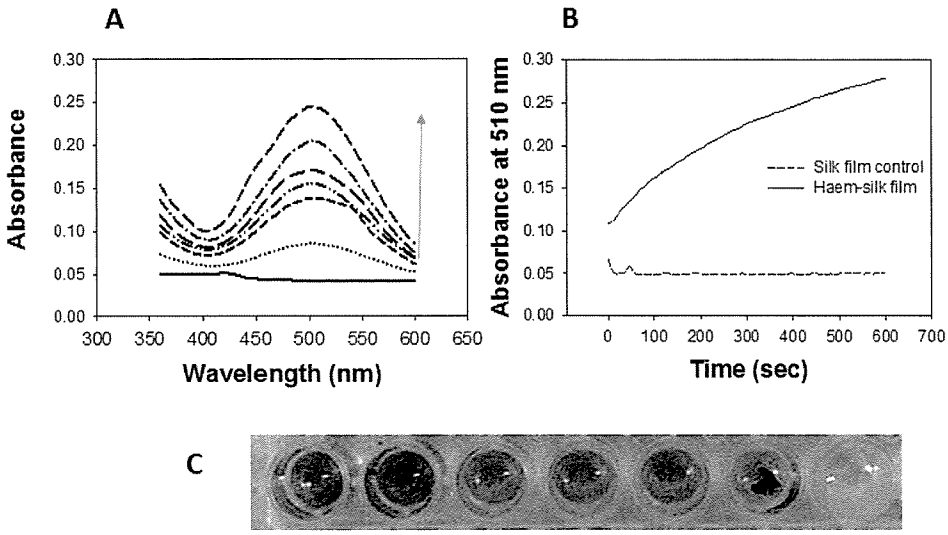


Figure 12

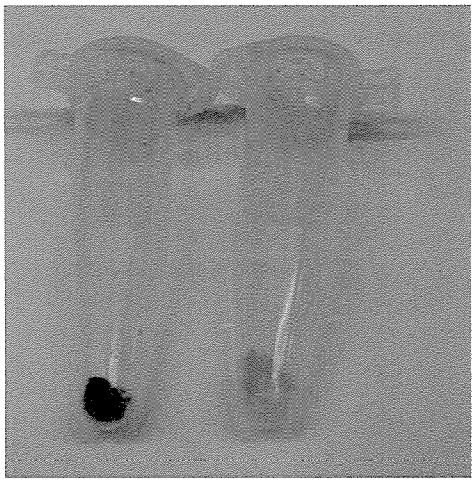


Figure 13

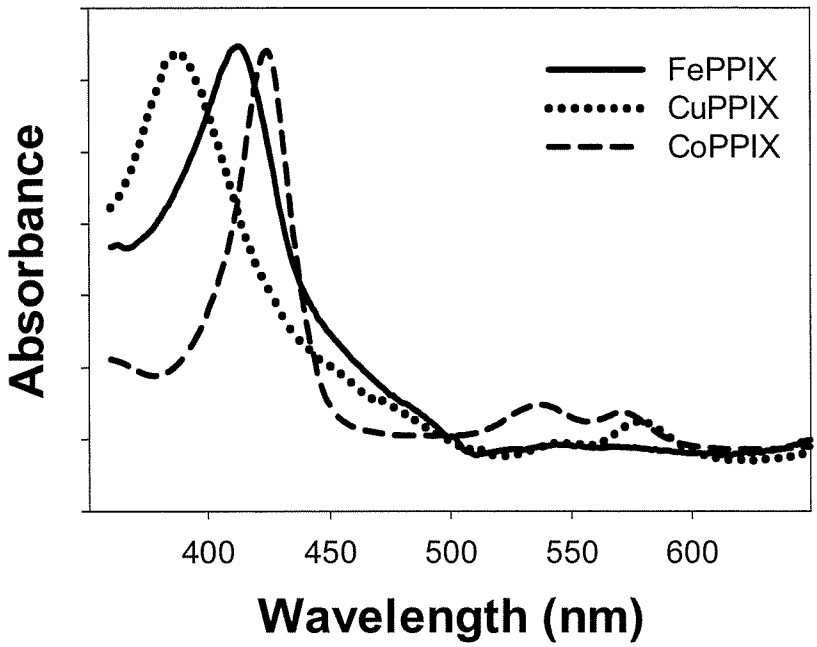


Figure 14

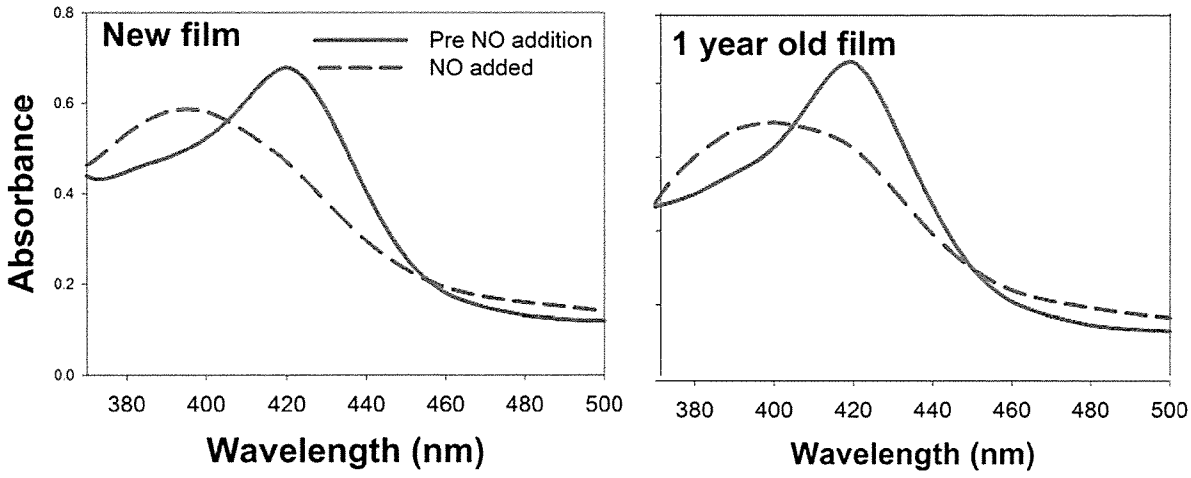
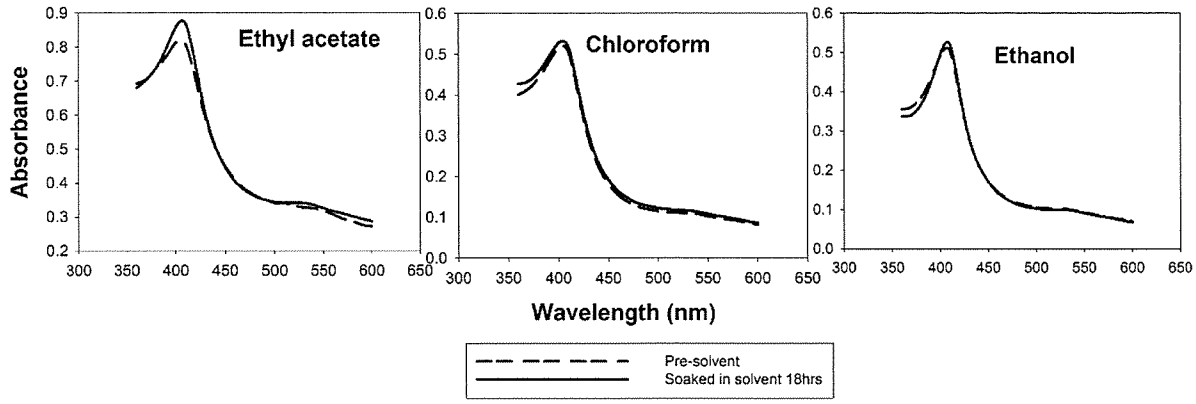


Figure 15

A



B

Figure 16

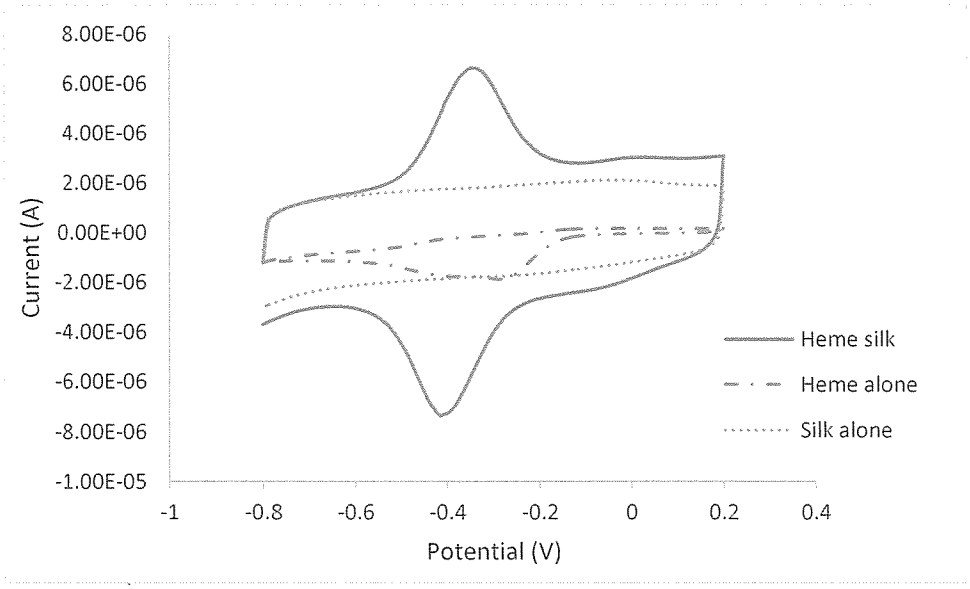


Figure 17

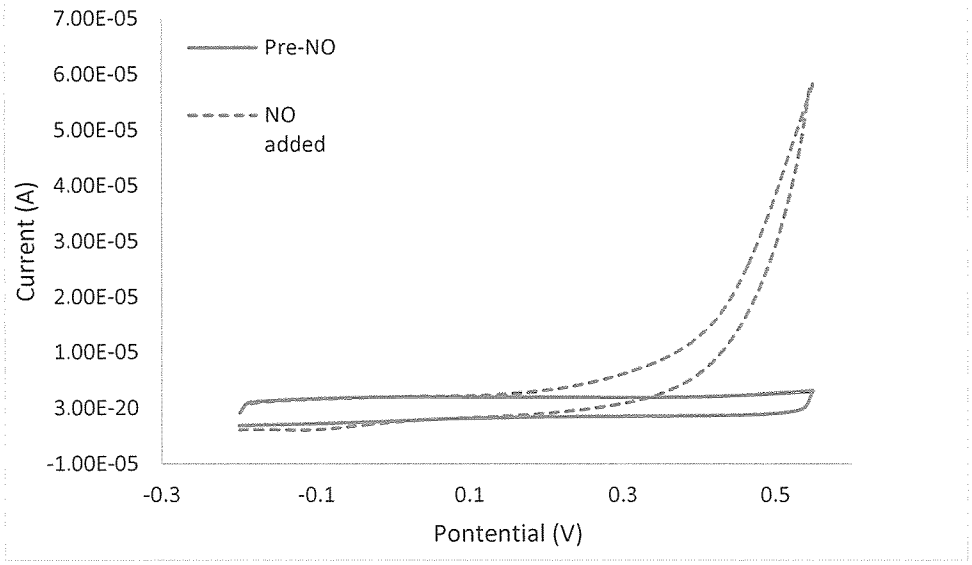


Figure 18

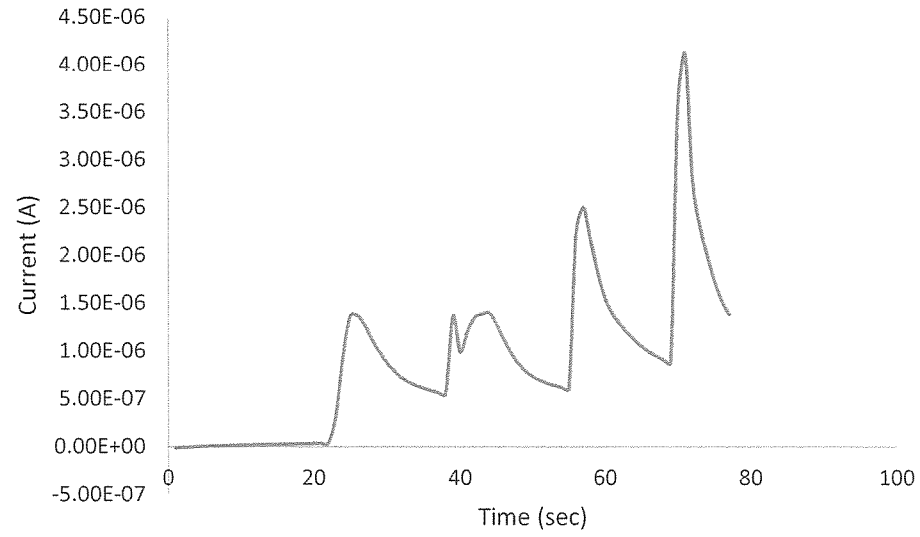


Figure 19

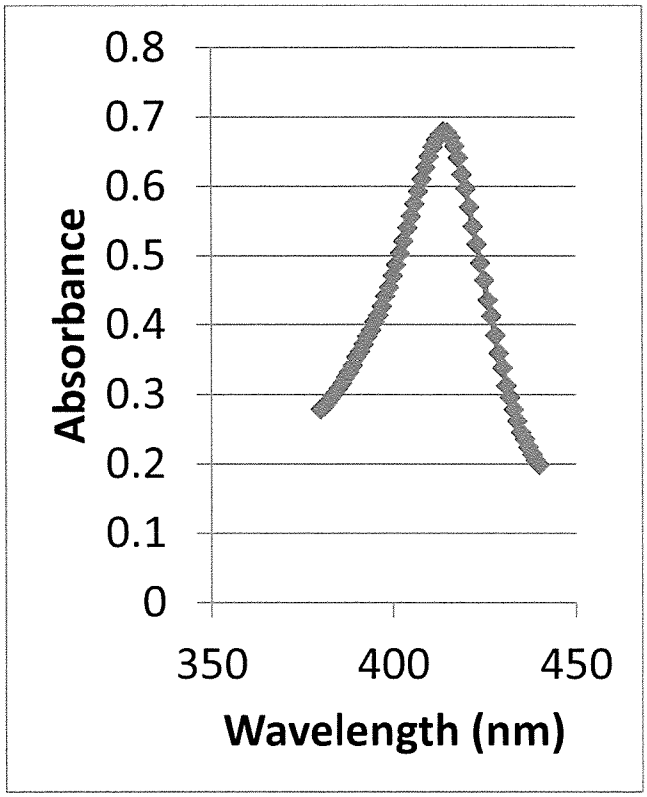
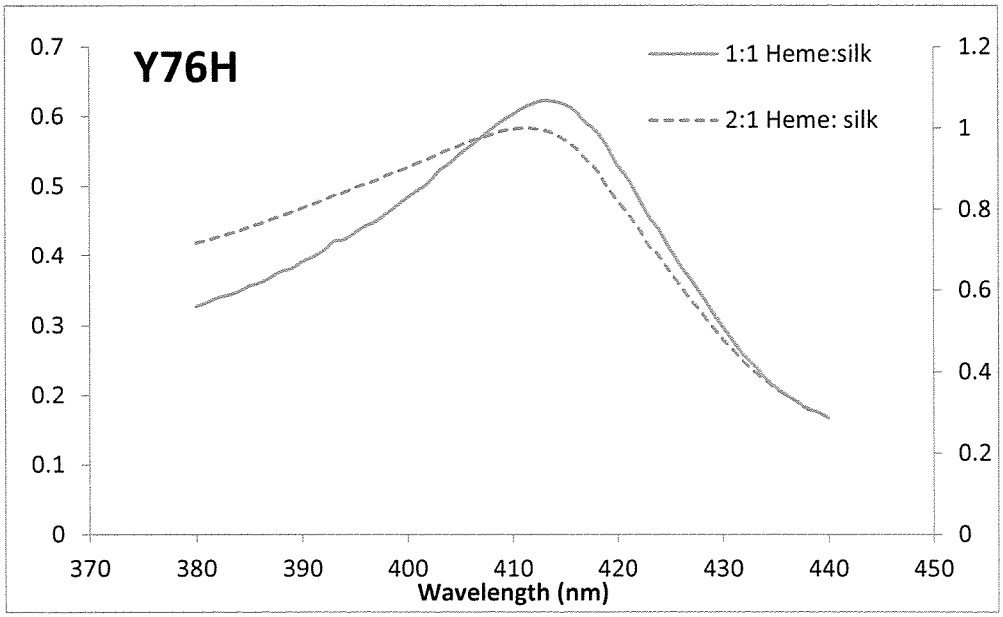
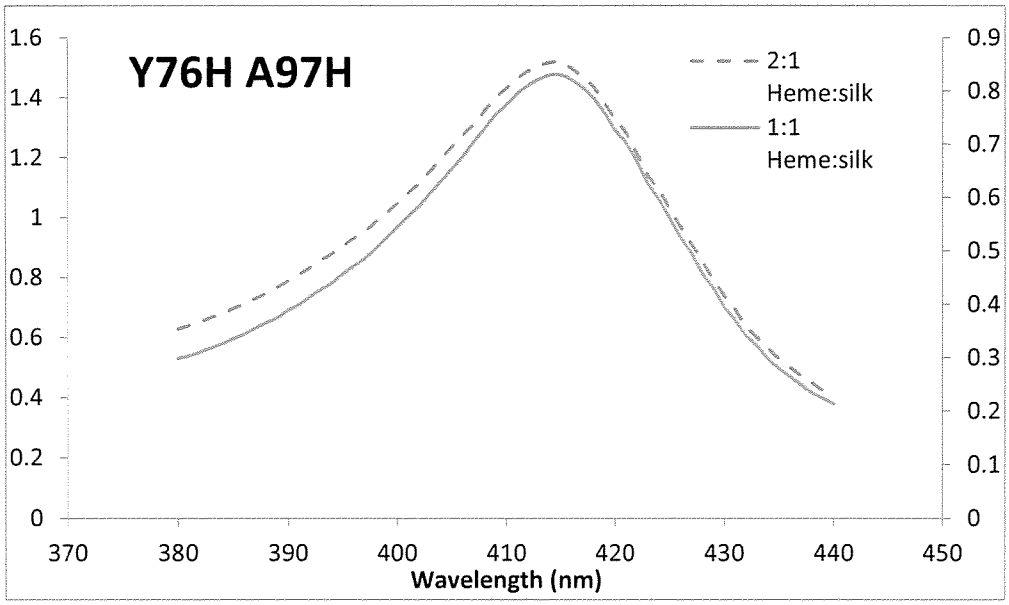


Figure 20

A


Y76H
1:1 Heme:silk
2:1 Heme: silk
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.2
1
0.8
0.6
0.4
0.2
0
370
380
390
400
410
420
430
440
450
Wavelength (nm)

B


Y76H A97H
2:1 Heme:silk
1:1 Heme:silk
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
370
380
390
400
410
420
430
440
450
Wavelength (nm)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/050717

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/795 (2006.01) D01F 4/02 (2006.01) C12Q 1/25 (2006.01) G01N 31/10 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPIAP, TXPEA, TXPEB, TXPEC, TXPEE, TXPEF, TXPEH, TXPEI, TXPEP, TXPES, TXPEPEA, TXPUSE0A, TXPUSE1A, TXPUSEA, TXPUSEB, TXPWOEA, MEDLINE, HCAPLUS, BIOSIS, BIOTECHABS, EMBASE, CAPLUS, GOOGLE SCHOLAR & Keywords: Coiled-coil, helix bundle, conformation, fibroin, insect, metalloprotein, metal chelate, chelant, cofactor, heme-silk, sensor, solid-state, G01N 31/00, G01N 33/00, and like terms;

EPODOC, WPIAP, MEDLINE, CAPLUS, BIOSIS, EMBASE, Google Scholar, AusPat, INTESS, PAMS & Keywords: Applicant and Inventor names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 2016	18 February 2016
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Alexander Theodossis AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 62832438

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. PCT/AU2015/050717
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAPSON, T.D. *et al.*, 'Micromolar biosensing of nitric oxide using myoglobin immobilized in a synthetic silk film', Biosensors and Bioelectronics, 2014, Vol. 62, pages 214-220 (Available online 26 June 2014) & Appendix A. Supplementary materials [online], [retrieved from the internet 11 February 2016] <URL: http://dx.doi.org/10.1016/j.bios.2014.06.045> Abstract; Sections 2.1, 2.2, 5; Figures 1, 2, 3; Scheme 1; Table 1; Suppl. Figure 1; para. bridging p.216-217; para.4 of col.2 on p.215; para.3 of col.1 on p.219	1-32
X	MCALLISTER, K.A. *et al.*, 'Using alpha-helical coiled-coils to design nanostructured metalloporphyrin arrays', Journal of the American Chemical Society, 2008, Vol. 130, pages 11921-11927 (pages 1-16 in viewed document retrieved from the internet) Abstract; Figures 1, 6; para.3 on p.6; para.3 on p.7; para.5 on p.8; Introduction; Conclusions	1-32
X	WO 2004/050693 A1 (NANOBIODESIGN LIMITED) 17 June 2004 Abstract; Figures 1, 3, 5; Tables 2, 3; lines 13, 14 on p.9; para.3 on p.3; para. bridging p.3-4; lines12, 13 on p.10	1-32
X	MUTTER, A.C. *et al.*, 'Rational design of a zinc phthalocyanine binding protein', Journal of Structural Biology, February 2014, Vol. 185, pages 178-185 Abstract; Figures 1, 5; para.4 of col.2 on p.181; Conclusions	1-32
X	WANG, C. *et al.*, 'Hybrid hydrogels assembled from synthetic polymers and coiled-coil protein domains', Nature, 1999, Vol. 397, pages 417-420 Abstract; Figures 1-3; para.2 of col.2 on p.418; para.2 of col.1 on p.419; para.1 of col.1 on p.418; para.2 of col.2 on p.419	1-32
X	KRISHNA, O.D. *et al.*, 'Protein- and peptide-modified synthetic polymeric biomaterials', Biopolymers, 2010, Vol. 94, pages 32-48 (pages 1-27 in viewed document retrieved from the internet) Abstract; Figure 3; para.2 on p.9	1-32
X	PAZOS, E. *et al.*, 'Sensing coiled-coil proteins through conformational modulation of energy transfer processes–selective detection of the oncogenic transcription factor c-Jun', Chemical Science, 2011, Vol. 2, pages 1984-1987 Abstract; Schemes 1, 2; Figure 2	1-32
X	YIN, H. *et al.*, 'Amperometric biosensor based on tyrosinase immobilized onto multiwalled carbon nanotubes-cobalt phthalocyanine-silk fibroin film and its application to determine bisphenol A', Analytica Chimica Acta, 2010, Vol. 659, pages 144-150 Abstract; Section 2.1	1-32
X	WO 2008/127402 A2 (TRUSTEES OF TUFTS COLLEGE) 23 October 2008 Abstract; para.46, 54, 55; Appendix II; Claims 1, 13	1-32
A	SUTHERLAND, T.D. *et al.*, 'Single honeybee silk protein mimics properties of multi-protein silk', PLoS One, 2011, Vol. 6, e16489 para.3 of col.2 on p.2; para. bridging col.1-2 on p.6	
	HUSON, M.G. *et al.*, 'Controlling the molecular structure and physical properties of artificial honeybee silk by heating or by immersion in solvents', PLoS One, 2012, Vol. 7, e52308 & NCBI Reference Sequence Accession NP_001129680 19 August 2013	

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2015/050717**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L	Supports Novelty Objection	
L	GLYKOS, N.M. *et al.*, 'Loopless Rop: structure and dynamics of an engineered homotetrameric variant of the repressor of primer protein', Biochemistry, 2006, Vol. 45, pages 10905-10919 Supports Novelty Objection	
P,X	RAPSON, T.D. *et al.*, 'De novo engineering of solid-state metalloproteins using recombinant coiled-coil silk', ACS Biomaterials Science & Engineering, 2015, Vol. 1, pages 1114-1120 Whole document	1-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/050717

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are 6 different inventions based on the following features that separate the claims into distinct groups:

Invention 1: Claims 1-32 (in part). The feature of a porphyrin chelating agent is specific to this group of claims.

Invention 2: Claims 1-32 (in part). The feature of a corrin chelating agent is specific to this group of claims.

Invention 3: Claims 1-32 (in part). The feature of a chlorin chelating agent is specific to this group of claims.

Invention 4: Claims 1-32 (in part). The feature of a corphin chelating agent is specific to this group of claims.

Invention 5: Claims 1-32 (in part). The feature of a porphine chelating agent is specific to this group of claims.

Invention 6: Claims 1-32 (in part). The feature of a phthalocyanine chelating agent is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is a composition comprising a metal chelate (a complex of a chelating agent and a metal ion) bound to a polypeptide having at least a partial coiled-coil structure. However this feature does not make a contribution over the prior art because it is disclosed in:

D2: MCALLISTER, K.A. *et al.*, 'Using alpha-helical coiled-coils to design nanostructured metalloporphyrin arrays', Journal of the American Chemical Society, 2008, Vol. 130, pages 11921-11927 (pages 1-16 in viewed document retrieved from the internet)

D7: PAZOS, E. *et al.*, 'Sensing coiled-coil proteins through conformational modulation of energy transfer processes–selective detection of the oncogenic transcription factor c-Jun', Chemical Science, 2011, Vol. 2, pages 1984-1987

D2 discloses a composition comprising a homotetrameric coiled-coil structure bound to iron-porphyrin cofactors (see Figures 1, 6).

D7 discloses a composition comprising a heterodimeric coiled-coil between c-Jun and an a-Fos construct, wherein the Tb-chelating macrocycle, DOTA, is bound to a-Fos (see Figure 2).

Therefore in light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

Notwithstanding the lack of unity, and following consultation with the applicant, the search was carried out in respect of all inventions listed above, with a focus on: a) silk fibroins; b) the concept of solid-state compositions comprising coiled-coil-forming polypeptides. In establishing the International Opinion all identified art relevant to the claims across their full scope has been considered.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2015/050717**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2004/050693 A1	17 June 2004	WO 2004050693 A1	17 Jun 2004
		AU 2003290221 A1	23 Jun 2004
		EP 1567548 A1	31 Aug 2005
		US 2006148026 A1	06 Jul 2006
WO 2008/127402 A2	23 October 2008	WO 2008127402 A2	23 Oct 2008
		CA 2704304 A1	23 Oct 2008
		CA 2704309 A1	20 Nov 2008
		CA 2704768 A1	23 Oct 2008
		CA 2704769 A1	23 Oct 2008
		EP 2086749 A2	12 Aug 2009
		EP 2086749 B1	08 May 2013
		EP 2101975 A2	23 Sep 2009
		EP 2107964 A2	14 Oct 2009
		EP 2109634 A2	21 Oct 2009
		EP 2612751 A2	10 Jul 2013
		EP 2650112 A2	16 Oct 2013
		JP 2010508852 A	25 Mar 2010
		JP 2010509593 A	25 Mar 2010
		JP 2010509644 A	25 Mar 2010
		JP 2010509645 A	25 Mar 2010
		JP 2013122462 A	20 Jun 2013
		JP 2014139684 A	31 Jul 2014
		US 2010063404 A1	11 Mar 2010

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2015/050717**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.